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## Appendices

## Appendix I

भारतसरकार
GOVERNMENT OF INDIA
पर्यावरण, वन और जलवायु परिवर्तन मंत्रालय
MINISTRY OF ENVIRONMENT, FOREST & CLIMATE CHANGE
भारतीय वनस्पति सर्वेक्षण
BOTANICAL SURVEY OF INDIA

दक्षिणीक्षेत्रीयकेन्द्र / Southern Regional Centre
टी,एन.ए.यूकैम्पस/ T.N.A.U. Campus
लाउलीरोड/ Lawley Road
कोयंबत्तूर/ Coimbatore - 641 003
टेलीफोन / Phone: 0422-2432788, 2432123, 2432487
टेलीफैक्स/ Telefax: 0422- 2432835
ई-मेल/E-mail id: sc@bsi.gov.in
bsisc@rediffmail.com

सं. भा.व.स./द.क्षे.के./No.: BSI/SRC/5/23/2021/Tech/241 दिनांक/Date: 25th February 2021

**पौधा प्रमाणीकरण प्रमाणपत्र /PLANT AUTHENTICATION CERTIFICATE**

The plant specimen which has been brought by you for authentication is identified as *Rhododendron arboreum* Sm. – ERICACEAE. The identified specimen is returned herewith for preservation in their College/ Department/ Institution Herbarium.

25/2/2021

डॉ.एम. यू. शरीफ़/Dr. M.U. Sharief
वैज्ञानिक 'ई' एवं कार्यालयाध्यक्ष/
Scientist 'E' &Head of Office
वैज्ञानिक 'ई' एवं कार्यालयाध्यक्ष
Scientist 'E' & Head of Office
भारतीय वनस्पति सर्वेक्षण
Botanical Survey of India
दक्षिणी क्षेत्रीय केन्द्र
Southern Regional Centre
कोयम्बत्तूर / Coimbatore - 641 003.

सेवा में / To

Ms. Yangchen Dolma Kom
Ph.D. Research Scholar
Department of Botany
Avinashilingam Institute for Home Science and Higher Education for Women
Coimbatore – 641 043, Tamil Nadu

## Appendix II

**Quantitative Estimation of Phytochemical Constituents**

**Estimation of Total Flavonoid Content** (Saeed *et al.,* 2012)

**Principle**

Total Flavonoid Content (TFC) was measured with the aluminium chloride colorimetric assay. Aluminium chloride forms acid stable complexes with the C-4 keto groups and either the C-3 or C-5 hydroxide group of flavones and flavonols. In addition it also forms liable complexes with ortho dihydroxide groups in A/B rings of flavonoids. For building the calibration curve, rutin was used as a standard material. Various concentrations of rutin solution were used to make a standard calibration curve.

**Reagents**

- Aluminium chloride ($AlCl_3$) - 0.3M
- Sodium nitrite ($NaNO_2$) - 0.5M
- Methanol - 30%
- Sodium hydroxide (NaOH) - 1M
- Stock standard solution: 100 mg Rutin was dissolved in 100 mL distilled water
- Working standard solution: 10 mL of the stock solution was made up to 100 mL by adding distilled water.

**Procedure**

In a 10 mL test tube, extracts of different concentrations (0.2,0.4,0.6,0.8,1) were taken and made up to 1 mL with distilled water. Add 3.4 mL of methanol to the test tubes. Followed by 0.15 mL of $NaNO_2$ and 0.15 mL of $AlCl_3.6H_2O$. After 5 min, 1 mL of NaOH was added. The estimation of the flavonoid compound was carried out in triplicates. The solution was mixed well and the absorbance was measured against a blank at 506 nm. The standard curve for total flavonoids was made using rutin as a standard solution (0 to 100 mg/L). The total flavonoids were expressed as milligrams of rutin equivalents per gram.

## Appendix III

**Estimation of Total Tannin Content** (Folin-Ciocalteu)

**Principle**

The reduction of a Phosphotungstic molybdic complex by tannin-like compounds in an alkaline solution and its estimation is based on the measurement of blue colour formed by reduction of Phosphotungstic molybdic acid. The intensity was measured in a spectrophotometer at 700 nm

**Reagents**

- Folin-Ciocalteau's reagent: One part of commercially available Folin-Ciocalteau's reagent was mixed with two parts of distilled water before use.
- Sodium carbonate (35 %)
- Stock Standard Solution: 100 mg tannic acid was dissolved in 100 mL distilled water.
- Working Standard solution: 10 mL of the stock solution was made up to 100 mL by adding distilled water.

**Procedure**

To 0.1 mL of sample extract 0.9 mL distilled water was added. To this mixture, 0.5 mL of Folin's reagent followed by 5 mL of 35 % sodium carbonate was added and kept at room temperature for 5 min. The experiment was repeated in triplicates. The same procedure was repeated for a standard solution and read against a blank. 1 mL of water served as blank without the sample. The blue colour formed was read at 700 nm using a UV/Visible spectrophotometer. The content of tannin was calculated using a standard graph and the result was expressed as mg TAE/g of fraction.

## Appendix IV

### Estimation of Total Alkaloid Content (Bromocresol green method)

**Principle**

The Alkaloids are naturally occurring nitrogen-containing pharmacologically active organic compounds present in plant kingdom. These have made major impact on plant medicine because of its vast application. The determination of Total Alkaloid Content (TAC) was based on the formation of yellow coloured complex by reaction between bromocresol green and alkaloid (Yoshida *et al.*, 2010) . The absorbance of the complex in chloroform was measured at spectrum in UV-Spectrophotometer.

**Reagents**

- Bromocresol green solution
- 2 N Hydrochloric acid
- Phosphate Buffer solution (pH 4.7)
- Stock Standard Solution: 100 mg Atropine was dissolved in 100 mL distilled water.
- Working Standard solution: 10 mL of the stock solution was made up to 100 mL by adding distilled water.

**Procedure**

A quantity of 0.2, 0.4, 0.6, 0.8 and 1 mL of working standard solution and 0.1 mL of the sample extract was taken in test tubes. About 5 mL of phosphate buffer (pH 4.7) was added. Then 5 mL of Bromocresol green solution was added. This mixture was added with 1, 2, 3 and 4 mL of chloroform. The absorbance of the complex in chloroform was read at 470 nm using a spectrophotometer against the blank prepared as above. Concentration was calculated using Atropine as standard and expressed as mg of AE/g of extract.

## Appendix V

**Estimation of Total Phenolic Content** (Chedea & Pop, 2019)

**Principle**

The method strongly relies on the reduction of the mixture heteropoly phosphotungstic-molybdates by the phenolic compound which results in the formation of blue colour chromogen. The phenolic compounds react with Folin-Ciocalteu reagent only under basic conditions adjusted by sodium carbonate solution. It has been observed that the phenolic compound undergoes dissociation to form a phenolate anion which reduces the Folin- Ciocalteu reagent i.e., the mixture of tungstate's and molybdates rendering a blue coloured solution. The colour intensity of the formed blue chromogen can be measured by the absorbance readings using a spectrophotometer. The products of the metal oxide reduction have a blue absorption with a maximum at 765 nm. The intensity of the light absorption at that wave length is proportional to the concentration of phenols. By using standard Gallic acid calibration curve, measure the concentration of phenolic content in Gallic acid total equivalents using units mg/g (GAE).

**Reagents**

- Folin-Ciocalteu's reagent.
- Sodium carbonate ($Na_2CO_3$)
- Stock Standard Solution: 100 mg Gallic acid was dissolved in 100 mL distilled water.
- Working Standard solution: 10 mL of the stock solution was made up to 100 mL by adding distilled water.

**Procedure**

1 mL of extract and different concentrations (0.2, 0.4, 0.6, 0.8, 1 mL) of gallic acid were taken and 1 mL of Folin-Ciocalteau's reagent was added to the test tubes. After 5 min, 10 mL of 7% $Na_2CO_3$ solution was added to the mixture followed by the addition of 13 mL of distilled water and mixed thoroughly. The estimation of the phenolic compound was carried out in triplicates. The mixture was kept in the dark for 90 min after which the absorbance was read at 750 nm.

## Appendix VI

**DPPH (2,2-Diphenyl-1-picrylhydrazyl) Radical Scavenging Assay** (BLOIS, 1958)

**Principle**

DPPH (2,2-Diphenyl-1-picrylhydrazyl) is a commercially available stable free radical, which is purple. The antioxidant molecules present in the herbal extracts, when incubated, react with DPPH and convert it into di-phenyl hydrazine, which is yellow. The degree of discoloration of purple to yellow was measured at 517 nm, which is a measure of the scavenging potential of plant extracts.

**Reagents**

- DPPH solution: 0.004 g of DPPH (0.004%) in 100 mL of methanol was made in a volumetric flask. Cover it with 2-3 layers of Aluminium foil and store it in the dark. Prepared fresh before use.
- Stock Standard Solution: Ascorbic acid was used as a standard. 10 mg of ascorbic acid dissolved in 10 mL of methanol. Dilutions of this solution with distilled water were prepared to give the concentration of 10, 25, 50, 100, 150 and 200 µL.
- Stock solutions of the sample were prepared by dissolving 10 mg of dried extract in 10 mL of methanol (1:1).

**Procedure**

Different concentrations of methanolic extract like 10, 25, 50, 100, 150 and 200 µL were taken in a series of test tubes. Made it up to 3 mL using methanol. 50 µL of DPPH solution to all the test tubes, shaken well and incubated in the dark for about 20- 30 minutes. Methanol serves as the control and DPPH in methanol solution acts as blank without plant extracts. The changes in the absorbance of the plant samples were measured at 517 nm using a spectrophotometer. Results were compared with the standard ascorbic acid. The ability of DPPH radical scavenging activity was calculated by using the following formula:

$$Pi = Ab - As \div Ab * 100\%$$

The results were expressed as % inhibited 1 mg of extract. The absorbance of the control (Ab) and the absorbance of the sample (As) were used to calculate the $IC_{50}$ values through linear regression analysis, which served to quantify the antioxidant capacity of the extract.

## Appendix VII

**ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) Radical Scavenging Assay** (Re *et al.,* 1999)

**Principle**

The antioxidant effect of the leaf and flower extracts were studied using ABTS (2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid) radical cation decolourization assay.

**Reagent**

- ABTS solution (7mM with 2.45mM Ammonium persulfate)
- Ethanol

**Procedure**

ABTS radical cations ($ABTS^{+}$) were produced by reacting ABTS solution (7mM) with 2.45 mM ammonium persulfate. The mixture was allowed to stand in the dark at room temperature for 12-16 hours before use. Aliquots (5 µL) of the leaf and flower extracts were added to 0.3 mL of ABTS solution and the final volume was made up to 1 mL with ethanol. The absorbance was read at 745 nm in a spectrophotometer and the percent scavenging was calculated using the formula:

$$Pi = As - Ac \div Ac * 100$$

The results were expressed as % inhibited 1 mg of extract. The absorbance of the control (Ac) and the absorbance of the sample (As) were used to calculate the $IC_{50}$ values through linear regression analysis, which served to quantify the antioxidant capacity of the extract.

## Appendix VIII

### $H_2O_2$ (Hydrogen Peroxide) Scavenging Activity

**Principle**

Peroxides containing oxygen–oxygen single bonds are unstable and decompose slowly when exposed to light. They are found in biological systems, including the human body. Peroxidases are enzymes that oxidize or decompose $H_2O_2$ . $H_2O_2$ is not a free radical, but instead an oxidizing agent, derived from the two electron reduction of $O_2$. A Fenton reaction can be used to produce OH from $H_2O_2$ in the presence of transition metal ions. The ability of the leaf and flower extracts to scavenge hydrogen peroxide was assessed by the method of Ruch *et al.,* (1989).

**Reagents**

- Phosphate buffer (0.1 M, pH7.4)
- $H_2O_2$ (40 mM) in phosphate buffer

**Procedure**

A solution of $H_2O_2$ (40 mM) was prepared in phosphate buffer. The leaf and flower extracts (5 µL) were added to $H_2O_2$ solution (0.6 mL) and the total volume was made up to 3 mL. The absorbance of the reaction mixture was recorded at 230 nm in a spectrophotometer. A blank solution containing phosphate buffer, without $H_2O_2$ was prepared. The extent of $H_2O_2$ scavenging activity of the plant extracts was calculated:

**Pi= As- Ac ÷ Ac * 100**

The absorbance of the control (Ac) and the absorbance of the sample (As) were used to calculate the $IC_{50}$ values through linear regression analysis, which served to quantify the antioxidant capacity of the extract.

## Appendix IX

**Lipid Peroxidation Assay (LPO)** (Repetto *et al.,* 2012)

**Principle**

The process by which free radicals steal electrons from lipids in the cell membrane, which results in cell damage. It involves a free radical chain reaction mechanism. Due to the presence of multiple double bonds in polyunsaturated fatty acids, methylene bridges (-$CH_2$-) with particularly reactive hydrogen atoms are most often impacted.

**Reagents**

- Egg lecithin
- Ferric Chloride
- 15% Trichloroacetic acid
- Thiobarbituric acid

**Procedure**

A 3 mg/mL of egg lecithin was prepared with phosphate buffer (pH 7.4) and it was sonicated and used for further study. 1 mL of various concentration of extracts was added with 1 mL of egg lecithin mixture. LPO was initiated by adding 10 µL (400 mm) of ferric chloride, 10 µL (200 mm) of ascorbic acid. Then this solution was kept for incubation for 1 hour at 37˚C, with this 2 mL of 0.25N HCl containing 15% TCA and 0.375% TBA was added. After that the mixture was boiled for 15 minutes, cooled and centrifuged to get clear supernatant. The supernatant was measured at 532 nm. The results were expressed as % inhibited 1 mg of extract.

**Pi = As- Ac ÷ Ac * 100**

The absorbance of the control (Ac) and the absorbance of the sample (As) were used to calculate the $IC_{50}$ values through linear regression analysis, which served to quantify the antioxidant capacity of the extract.

## Appendix X

**Ferric Reducing Antioxidant Power Assay (FRAP)** (Benzie & Strain, 1996).

**Principle**

FRAP (Ferric Reducing Antioxidant Power) assay is a widely used method in antioxidant research to assess the antioxidant capacity of various substances. The higher the FRAP value, the greater the antioxidant potential of the substance being tested. Substances, which have the reduction potential react with ferric ions ($Fe^{3+}$) to form ferrous ions ($Fe^{2+}$), which then reacts with ferric chloride to form ferric-ferrous complex that has the adsorption maximum at 700 nm.

**Reagent**

- The working FRAP reagent was prepared by mixing acetate buffer 300 mM (pH 3.6), 1 g sodium acetate trihydrate, 16 mL of glacial acetic acid and making the volume to 100 mL with distilled water.
- Then TPTZ (2, 4, 6-tripyridyl-s- triazine): (M.W. 312.34), 10 mM in 40 mM HCl (M.W. 36.46).
- $FeCl_3$. 6 $H_2O$: (M.W. 270.30), 20 mM was taken.
- All three (1,2,3) were taken in the ratio of 10:1:1 before testing.
- Standard was $FeSO_4$. 7 $H_2O$: 0.1 - 1.5 mM in methanol.

**Procedure**

Immediately after preparing the FRAP reagent, 3.6 mL was added to distilled water (0.4 mL) and was incubated at 37 ℃ for 5 min. This solution was mixed immediately with different concentration of the solution of *R. arboreum* leaf and flower (10, 20, 40, 60, 80, 100 and 200 µg/mL) and incubated at 37℃ for 10 min. The absorbance of the *R. arboreum* leaf and flower was measured at 593 nm. For construction of the calibration curve, seven concentrations of $FeSO_4$, $7H_2O$ (0.1, 0.4, 0.8, 1, 1.12, 1.5, 2.5 mM) were used and the absorbance values were measured as for sample solutions.

**Formula**

**FRAP value = As – intercept ÷ slope**

The intercept minus the absorbance of the sample (As) divided by slope were used to calculate the $IC_{50}$ values through linear regression analysis, which served to quantify the antioxidant capacity of the extract.

## Appendix XI

**Avinashilingam Institute for Home Science and Higher Education for Women**

Deemed to be University Estd. u/s 3 of UGC Act 1956, Category A by MHRD
Re-accredited with A++ Grade by NAAC. CGPA 3.65/4, Category I by UGC
**Coimbatore - 641 043, Tamil Nadu, India**

**(Reg. No. 623/PO/ReBi/S/02/CCSEA)**

# Certificate

This is to certify that the project proposal no **AIW:IAEC.2023:11** entitled **Anticancer activity of methanolic extracts of *Rhododendron arboreum* Sm. Leaves and flowers** submitted by **Ms. Yangchen Dolma Kom** has been approved/recommended by the IAEC of **Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore** (Organization) in its meeting held on **14/03/2023** (date) and **30 Albino rats (Wistar) C57BL/6** (Number and Species if animals) have been sanctioned under this proposal for a duration of next **12** months.

| Authorized by | Name | Signature | Date |
|---|---|---|---|
| Chairman: | Dr. Anitha Subash | ..... | 14/3/2023 |
| Member Secretary: | Dr. R. Nirmaladevi | ..... | 14/3/2023 |
| Main Nominee of CCSEA: | Dr. V. M. Berlin Grace | ..... | 14-03-2023 |

## Appendix XII

**Estimation of Haematological Parameters**

**(i) Enumeration of Red Blood Cells (RBCs) (**Raabe *et al.,* 2011)

**Principle**

The enumeration of red blood cells (RBCs) involves counting these cells per unit volume of blood, usually expressed as cells per cubic milli meter ($mm^3$) or litre. The process relies on the principles of dilution and microscopy.

**Procedure**

Using a red blood cell pipette, a well-mixed blood sample was drawn up to the 0.5 mark, and RBCs diluting fluid was added up to mark II. The mixture of fluid and blood was gently shaken and transferred onto the counting chamber. After allowing the cells to settle for 2 minutes without letting the fluid dry, RBCs were counted under a 45X or higher power objective, uniformly focusing on the larger corner squares of the chamber. The results were expressed as the number of cells x $10^{12}$ per liter (cells x $10^{12}$/L).

**(ii) Enumeration of White Blood Cells (WBCs)** (Gorva *et al*., 2022)

**Principle**

The principle of enumerating WBCs involves counting and quantifying the number of cells present in a specified volume of blood. This process is crucial for assessing the body's immune response and detecting abnormalities related to infections, inflammations or haematological disorders.

**Procedure**

Using a white blood cell pipette, draw a well-mixed blood sample up to the 0.5 mark. Add WBCs diluting fluid up to mark II. Gently shake the mixture of fluid and blood, then transfer it to the counting chamber. Allow the cells to settle to the chamber's bottom for 2 minutes, ensuring the fluid does not dry out. Using a 10 X or low-power objective, count the WBCs uniformly in the larger corner squares of the chamber. The results are expressed as the number of cells per 10 $mm^3$ (cells/10 $mm^3$).

**(iii) Differential Leukocyte Count** (Nivedhita *et al.,* 2020)

**Principle**

Differential leukocyte count, also known as White Blood Cell (WBCs) differential count, involves identifying and quantifying the different types of white blood cells present in a blood sample. This is typically done by examining a stained blood smear under a microscope, where different types of WBCs can be distinguished based on their size, shape and staining characteristics.

**Procedure**

Prepare blood films from anticoagulated blood specimens and stain them with Leishmann's stain. Examine the stained blood films under oil immersion microscopy to identify different types of white blood cells (WBCs). Determine the percentage distribution of these cells by counting them per 100 cells observed.

Calculate the absolute lymphocyte and neutrophil counts using the differential leukocyte count and total WBCs count obtained from the blood film examination.

$$\text{Absolute neutrophil count} = \frac{\text{Number of neutrophils}}{100} \times \text{TWBC}$$

$$\text{Absolute lymphocyte count} = \frac{\text{Number of lymphocytes}}{100} \times \text{TWBC}$$

**(iv) Estimation of Haemoglobin: Sahil's Acid Haematin Method** (Thakkar *et al.,* 2021)

**Principle**

Haemoglobin is converted into acid haematin using HCl. The resulting acid haematin solution is then diluted with distilled water until its colour matches that of a permanent standard in a comparator block. The concentration of haemoglobin (Hb) is determined directly from the calibration tube.

**Procedure**

Add 0.1 N HCl solution into the Sahil's Hemoglobinometer up to the lowest marking using a pipette. Draw 20 µL of blood into the Sahil's pipette

up to the 20 µL mark. Carefully adjust the blood column in the pipette without introducing bubbles. Wipe off excess blood on the sides of the pipette using a dry piece of cotton. Blow the blood into the acid solution in the graduated tube of the Hemoglobinometer. Rinse the pipette thoroughly. Mix the reaction mixture well and allow it to stand at room temperature for 10 minutes. Dilute the solution with distilled water by carefully adding a few drops at a time, mixing the reaction mixture until the colour matches that of the comparator block. The lower meniscus of the fluid are recorded in grams per 100 mL.

## Appendix XIII

**Estimation of Serum Biochemical Parameters**

**(i) Determination of Total Proteins** (Waterborg *et al.,* 2009)

**Principle**

This method is a combination of both Folin-Ciocalteu and Biuret reaction which involves two step reaction. In the first Step Protein binds with copper in alkaline medium and reduces it to Cu++. In the second step Cu++ formed catalyses the oxidation reaction of aromatic amino acid by reducing Phosphomolybdotungstate to heteropolymolybdanum, which leads to the formation of blue colour and is measured at 640 nm.

**Procedure**

To 0.1 mL of the liver homogenate, 0.9 mL of water, 4.5 mL of alkaline copper sulphate reagent were added and allowed to stand in the room temperature for 10 min. To this 0.5 mL of Folin's reagent was added. After 20 min, the blue colour developed was measured at 640 nm. The level of protein present was expressed as mg/g tissue or mg/dL.

**(ii) Determination of SGOT and SGPT**

Transaminase level in serum was measured by using Ambica diagnostic kit. The kit utilize the colorimetric procedure of Reitman and Frankel in which the oxaloacetate and pyruvate formed in either the GOT or GPT reaction is combined with 2, 4- initrophenylhydrazine to yield a brown- coloured hydrazine which is measured at 505 nm (Reitman & Frankel, 1957).

**(iii) Determination of Alkaline Phosphatase activity (ALP)**

Alkaline Phosphatase converts phenyl phosphatase to inorganic phosphatase and phenol at pH 10.0. Phenol reacts in alkaline medium with 4-aminoantipyrine in the presence of the oxidizing agent potassium ferricyanide and forms an orange red coloured complex, which can be measured calorimetrically. The colour intensity is proportional to enzyme activity (Marsh *et al.,* 1959).

**Procedure**

Initially buffered substrate with distilled water was incubated for 3 min at 37$^{o}$C. the phenol standard (10 mg%) / tissue homogenate/ serum were added. Mixed well and incubated for 15 min at 37$^{o}$C then chromogen reagent was added and OD was measured at 510 nm.

**Calculation:**

Alkaline Phosphatase activity in KA units = OD test/ OD standard X 10

**(iv) Determination of Acid Phosphatase (ACP)**

Total acid phosphatase activity was measured by the colorimetric method described by Lin & Fishman, 1972.

The compound, p-nitrophenyl phosphate is colourless but upon hydrolysis the phosphate group, the yellow compound of p-nitrophenyl is liberated. Thus, the substrate itself acts as an indicator of the amount of splitting and hence is a measure of phosphate activity as indicated by the following reaction. The reaction is catalysed by an acid phosphatase at pH 4.8. The intensity of the colour produced is directly proportional to the acid phosphatase activity in the sample. Absorbance of serum blank, total acid phosphatase tubes were taken against distilled water on shimadzu UV 160 instrument at 405 nm wavelength within 20 minutes. If the absorbance of the test sample exceeded 0.8800, estimation was repeated by diluting 0.2 mL of the serum sample with 0.6 mL of 0.9% NaCl solution and repeat the assay. The results were multiplied by 4 (the dilution factor).

**Calculation:**

ACP U/L (units/Litre) = Absorbance of sample X factor / Time (minutes).

## Appendix XIV

**Renal Function Parameters**

**(i) Determination of Urea**

Urea level in serum was measured by using Ambica diagnostic kit. The kit utilize the colorimetric procedure in which urease catalyses conversion of urea to ammonia and carbon dioxide. The ammonia then reacts with a mixture of salicylate, hypochlorate and nitroprusside to yield a blue-green coloured compound (indophenols). The intensity of colour produced is proportional to the concentration of urea in the sample and is measured photometrically at 578 nm (Bergmeyer, 1965).

**(ii) Determination of Uric acid (Holmes & Assimos, 2004)**

Serum uric acid was estimated in deproteinized diluted serum. To 0.5 mL of serum, 3.5 mL of distilled water was added followed by 0.5 mL of 2/3 N $H_2SO_4$ and 0.5 mL of 10 % sodium tungstate. After through mixing the tubes were centrifuged. To 3.0 mL of phosphotungstic acid reagent. A blank and a standard were simultaneously prepared. Standard received 50 mg of uric acid. The optical density was read at 660 nm

**Calculation:**

Reading of the test sample/ reading of the standard= mg Uric acid

**(iii) Determination of Creatinine**

Creatinine level in serum was measured by using Ambica diagnostic kit. The kit utilize the colorimetric procedure of Haffe in creatinine in alkaline medium reacts with picric acid to produce a red coloured complex. The rate of this reaction measures creatinine concentration at 505 nm. After complete colour development, the absorbance is measured and the medium is acidified with acid reagent. The colour developed by true creatinine fades rapidly whereas that of non-creatinine chromogens remains unaffected. Hence this difference in absorbance is directly proportional to the true creatinine concentration (Chaney & Marbach, 1962).

## Appendix XV

**Enzymatic and Non- Enzymatic Antioxidant Markers**

**(i) Determination of Total Proteins** (Waterborg *et al.,* 2009)

**Principle**

This method is a combination of both Folin-ciocalteau and Biuret reaction which involves two step reaction. In the first Step Protein binds with copper in alkaline medium and reduces it to $Cu^{++}$. In the second step $Cu^{++}$ formed catalyses the oxidation reaction of aromatic amino acid by reducing Phosphomolybdotungstate to heteropolymolybdanum, which leads to the formation of blue colour which is measured at 640 nm.

**Procedure**

To 0.1 mL of the liver homogenate, 0.9 mL of water, 4.5 mL of alkaline copper sulphate reagent were added and allowed to stand in the room temperature for 10 min. To this 0.5 mL of Folin's reagent was added. After 20 min, the blue colour developed was measured at 640 nm. The level of protein present was expressed as mg/g tissue or mg/dL.

**(ii) Determination of Superoxide Dismutase (SOD)** (Kakkar *et al.,* 1984)

**Principle**

Superoxide dismutase scavenges the superoxide radical ($O^{\bullet}_2$) and thus provides a first line defence against free radical damage. Superoxide dismutase is an endogenous enzymatic antioxidant which catalyzes the dismutation of superoxide free radical. This method is based on the inhibition of the spontaneous oxidation of the adrenaline to adrenochrome by the enzyme superoxide dismutase.

$$2H_2O + 2O_2^- \xrightarrow{\text{Superoxide dismutase}} 2H_2O_2 + O_2$$

Superoxide anion ($O_2^-$) interacts with peroxide to form hydroxyl radical ($OH^{\bullet}$) which causes damage in the absence of superoxide dismutase activity.

**Procedure**

Liver homogenate (0.5 mL) was diluted with 0.5 mL of distilled water. To this, 0.25 mL ethanol and 0.15 mL of chloroform, all reagents kept for chilled and then were added. The mixture was shaken for 1 minute and centrifuged at

2000 rpm. The enzyme in the supernatant was determined. To 0.5 mL of the supernatant, 1.5 mL of buffer was added. The reaction was initiated by the addition of 0.4 mL epinephrine and change in optical density per minute was measured at 480 nm in a double beam UV-VIS spectrophotometer (UV 1700, Shimadzu) SOD activity was expressed as U/mg. Change in optical density per minute at 50% inhibition to adrenochrome transition by the enzyme is taken as one enzyme unit.

**(iii) Determination of Catalase (CAT)** (Sinha *et al.,* 2022)

**Principle**

In animals, catalase is present in all major body organs, especially being concentrated in liver and erythrocyte. During β-oxidation of fatty acids by flavoprotein dehydrogenase, hydrogen peroxide is generated, which is accepted upon by Catalase present in peroxisomes. Catalase catalyses the rapid decomposition of hydrogen peroxide to water.

$$2H_2O_2 \xrightarrow[\text{Catalase}]{} 2H_2O + O_2$$

Dichromate in acetic acid was converted to perchloric acid and then to chromic acetate when heated in presence of hydrogen peroxide. The chromic acetate thus produced is measured spectrophotometrically at 610 nm. The reaction is stopped at specific time interval by the addition of dichromate-acetic acid mixture and the remaining hydrogen peroxide is determined by measuring chromic acetate.

**Procedure**

Liver homogenate (0.1 mL) was taken, to which 1.0 mL of phosphate buffer and hydrogen peroxide were added. The reaction was arrested by the addition of 0.2 mL dichromate acetic acid reagent. Standard hydrogen peroxide in the range of 4 to 20 µL were taken and treated similarly. The tubes were heated in a boiling water bath for 10 min. The green colour developed was read at 570 nm in a Double beam UV-VIS spectrophotometer (UV 1700, Shimadzu). Catalase activity was expressed as U/mg.

**(iv) Determination of Glutathione Peroxidase (GPx)** (Rotruck *et al*., 1973)

**Principle**

Glutathione peroxidase catalyses the following reaction.

$$R\text{-}COOH + 2GSH + O_2 \xrightarrow{\text{Glutathione peroxide}} R\text{-}CH(OH)\text{-}COOH + GSSG + H_2O$$

Glutathione was measured by its reaction with DTNB to give a compound that absorbs at 412 nm.

**Procedure**

EDTA (0.2 mL each), sodium azide, reduced glutathione, $H_2O_2$; 0.4 mL of buffer and 0.1 mL of enzyme (liver homogenate) were mixed and incubated at 37°C for 10 min. The reaction was arrested by the addition of 0.5 mL of TCA and the tubes were centrifuged. To 0.5 mL of supernatant, 3 mL of sodium hydrogen phosphate and 1 mL of DTNB were added and the colour developed was read at 412 nm immediately in a Double beam UV-VIS spectrophotometer/ UV 1700, Shimadzu. Glutathione peroxidase activity, in serum is expressed as µg/mg.

**(v) Determination of Reduced Glutathione (GSH)** (Ellman,1959)

**Principle**

DTNB (5, 5'-dithiobis (2-nitrobenzoic acid)), known as Ellman's Reagent, was used for the detection of thiol compounds. DTNB and glutathione (GSH) react to generate 2-nitro-5-thiobenzoic acid and glutathione disulphide (GSSG), where 2-nitro-5-thiobenzoic acid yield a stable yellow colored product, which is proportional to GSH concentration, measured at 412 nm.

**Procedure**

To 250 µL of tissue homogenate taken in 2 mL eppendorf tube, 1 mL of 5% TCA was added and the above solution was centrifuged at 3000 rpm for 10 min at room temperature. To 250 µL of the above supernatant, 1.5 mL of 0.2 M phosphate buffer was added and mixed well. 250 µL of 0.6 mM of Ellman's reagent (DTNB solution) was added to the above mixture and the absorbance was measured at 412 nm within 10 min. A standard graph was plotted using glutathione reduced solution (1 mg/mL) and GSH content present

in the tissue homogenates was calculated by interpolation. Amount of glutathione expressed as μg/mg protein.

**(vi) Determination of Lipid Peroxidation (LPO)** (Okhawa *et al*., 1979)

**Principle**

In this method malondialdehyde and other TBARS (Thiobarbituric acid reactive substances) were estimated by their reactivity with thiobarbituric acid TBA) in acidic condition to generate a pink coloured chromophore which were read at 535 nm.

**Procedure**

1 mL of liver homogenate was mixed with 0.2 mL 4 % (w/v) sodium dodecyl sulfate, 1.5 mL 20% acetic acid in 0.27 M hydrochloric acid (pH 3.5) and 15 mL of 0.8% thiobarbituric acid (TBA, pH 7.4). The mixture was heated in a hot water bath at 85˚C for 1 hour. The intensity of the pink colour developed was read against a reagent blank at 532 nm following centrifugation at 1200 rpm for 10 min. The concentration was expressed as *n* moles of MDA per mg of protein using 1,1,3,3,-tetra-ethoxypropane as the standard.

## Appendix XVI

### Structure and Bioavailability Radar

### Molecular size

It is the assess of the area a substance occupies in 3D space. The total space any mass takes up in 3D space is known particularly as its volume. The drug-like molecule have a molecular size of 200-600g/mol (Wildman & Crippen, 1999).

### Flexibility-Rotatable bonds

The number of rotatable bonds is the number of bonds that allow free rotation around themselves. These are defined as every single bond, not in a ring and bound to a non-terminal heavy atom. Carbon nitrogen bonds of amides are omitted from the count due to their high rotational barrier. The drug-like molecule has several rotatable bonds < 15 (Egan *et al.,* 2000).

### Polarity (TPSA)

Polarity is the separation of electrical charge leading to a molecule or its chemical groups having an electric dipole moment with a negatively charged and positively charged end. The reduced TPSA correlates increased permeation rate. The drug-like molecule has a TPSA of < 150 (Lombardo *et al.,* 2003).

### H bond acceptors and H bond donor

Hydrogen bonds are formed between hydrogen atoms bound to a small, strongly electro-pulling atom with an unshared pair of electrons. Aliphatic fluorine, oxygen and nitrogen are Hydrogen bond acceptors and all nitrogen and oxygen atoms with at least one hydrogen are hydrogen bond donors. The drug-like molecule has an hydrogen bond acceptor ≤ 10 and an hydrogen bond donor ≤ 5 (Di *et al.,* 2012).

### Molar refractivity

It measures the total polarisability of a mole of a substance and depends on the temperature, the refractive index and the pressure. The drug-like molecule has a molar refractive index of 40-130 (Brito *et al.,* 2015).

### Lipophilicity

Lipophilicity is one of the most important parameters in the discovery and development of drugs, which are experimentally demonstrated as partition coefficients (log P) or distribution coefficients (log D). Log P indicates the

partition equilibrium of a non-ionised solute in the middle of water and an immiscible organic solvent. The greater the log P values, the greater the lipophilicity (Moriguchi *et al.,* 1994). The following five models provided by Swiss ADME to determine the character of the lipophilicity of a compound are as follows

- **XLOGP3**- It is an atomistic approach with correction factors and an information-based library. The drug-like molecule has XLOGP between -2 and 5.
- **WLOGP** - It is the application of a purely atomistic method based on a fragmentary system.
- **MLOGP**- It is an archetype of the topological method based on a linear relationship with 13 implemented molecular descriptors.
- **SILICOS-IT**- It is an mongrel method entrust on 27 fragments and 7 topological descriptors.
- **iLOGP**- It is a physics based method that relies on the free energies of solvation in n-octanol and water calculated with the generalised born and solvent accessible surface area (GB/SA) model. The consensus log P o/w is an arithmetic mean of the values predicted by the five proposed methods (Darvas *et al.,* 2002).

**Gastro Intestinal absorption**

Drugs are absorbed from the gastrointestinal tract by passive non-ionic diffusion or active transport. Passive diffusion is the most important mechanism and essentially depends on the movement of drugs through the mucosa into the bloodstream along a concentration gradient. The transfer rate depends on the concentration gradient, molecular weight, lipid solubility and mucosal perfusion, surface area and permeability. The epithelial cells act as a lipid membrane, permeable to lipid-soluble compounds but relatively impermeable to highly ionised water-soluble substances.

**Blood Brain Barrier (BBB) permeation**

BBB penetration is a parameter used to determine whether the drug crosses the blood-brain barrier. Normally, most drugs are not allowed to cross the blood-brain barrier if the target is not associated with the nervous system.

**CYP2D6 inhibitor**

CYP2D6, a member of the cytochrome P450 oxidase system with multifunction, is one of the most significant enzymes associated in the metabolism of xenobiotics in the body. In primarily, CYP2D6 is responsible for the metabolism and excretion of approximately 25% of clinically used drugs by adding or removing certain functional groups – notably hydroxylation, demethylation and dealkylation.CYP2D6 also activates some prodrugs. This enzyme also metabolises several endogenous substances, such as hydroxytryptamines, neurosteroids and m-tyramine and p-tyramine, which CYP2D6 converts to dopamine in the brain and liver (Ogu & Maxa, 2000).

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# Appendix L2
## (Item No 5 of Check List)
## Details of Research Publications

| S.No | Article | Journal | Other Details Vol/No/Page No/ Year | Published in UGC- CARE / Scopus Indexed/ Web of Science |
|---|---|---|---|---|
| 1 | Evaluation of anticancer potential of methanolic leaf and flower extracts of *Rhododendron arboreum* Sm in AGS gastric adenocarcinoma cell line | Medicinal Plants- International Journal of Phytomedicines and Related Industries | Vol no-15 Issue 4 Pg no- 776-783 2023 | Scopus Indexed |
| 2 | Phytochemical Profiling and Antioxidant Evaluation of *Rhododendron arboreum* Sm leaf and flower: Integrative Analysis using Advanced Analytical Techniques | Drug Development and Industrial Pharmacy | Vol no-50 Issue 7 Pg no. 687-705 2024 | Scopus Indexed/ Web of Science |
| 3 | A Review: *Ophiocordycep Sinensis* (Berk.) as a Traditional Tibetan Medicine and its Potential in the Treatment of Various Human Ailments | Current Traditional Medicine | Vol no- 10 Issue 6 Pg- 1-8 2024 | Scopus Indexed |

*Proof of list of Journals from the Internet to be attached along with copies of reprints.

Scholar :

Supervisor :

Library have checked the papers

HoD 3/9/24

Checked By:

3/9/2024

Dean of Respective School

The scholar Miss Yangchen Dolma Kom (19PHBOF004) has published her research articles in the following journals :

1. Medicinal Plants : International Journal of Phytomedicines and related Industries- indexed in Scopus
2. Drug Developmet and Industrial Phasmacy - indexed in Scopus

This may be considered

03-09-24.

Asst. Librarian

doi : 10.5958/0975-6892.2023.00079.5

**Medicinal Plants**
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**Research Article**

# Evaluation of anticancer potential of methanolic leaf and flower extracts of *Rhododendron arboreum Sm* in AGS gastric adenocarcinoma cell line

**Yangchen Dolma Kom and R. Karthiyayini***
*Department of Botany, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore-641043, Tamil Nadu, India*

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**ABSTRACT**

This study aimed to determine the anticancer potential of methanolic extract of flowers and leaves (RFM, RLM) of *Rhododendron arboreum* Sm by cytotoxicity and lethality assays. The shrimps in the sample (RFM, RLM) were found to be significantly less toxic in lower and higher concentrations (100, 250, 500, 1000, 1500 µg/ml). Even after 24 h, 1-4 shrimps were observed mortal at the highest concentration, resulted in vigorous cytotoxic activity of methanol extract of flowers, with an $IC_{50}$ value of 0.541 mg/ml. At the same time, the cytotoxic effects of methanol leaf extract showed comparatively lower cytotoxic effects on AGS cells with $IC_{50}$ value of 0.841 mg/ml. Low toxicity on normal cells was demonstrated by both the RFM and RLM by the $LC_{50}$ values, which suggested that both extracts can be considered for anticancer potentials by conducting future experiments.

**Keywords:** Brine shrimp assay, flow cytometry, mortality, MTT, potassium dichromate and propidium iodide

## INTRODUCTION

India is considered the world's largest producer of medicinal herbs due to its rich biodiversity of 2,500 species of medicinal plants, among which the WHO has listed 21,000 plants (Rajasekharan *et al.,* 2020). It was impossible to imagine the scientific evidence, philosophical and experimental bases, molecules underlying the medicinal properties of herbs or herbal genomics when the medicinal properties of herbs were discovered (Chakraborty, 2018). Alternative and complementary medicine is experiencing constant and renewed interest primarily due to high costs, side effects, microbial resistance and the lack of curative treatments for a variety of chronic diseases (Cardos *et al.,* 2021). Global data indicates that 80% of the world's population uses ethnobotanical remedies and plant drugs primarily (Tefera *et al.,* 2019). As of late 2007, almost 100 plant-derived compounds were used in clinical trials, but even though plant-derived natural products remain abundant resources for drug development and have had a profound and lasting impact on human health, their clinical potential is often limited by low production levels in plants and loss of resources for extinction (Chakraborty *et al.,* 2018). It would be of more interest to know *Rhododendrons'* genetic diversity and genomic resources to preserve and promote sustainable resource usage. *Rhododendrons* are essential components of alpine and subalpine vegetation, presently encountering extinction under current climate change and high frequency of habitat disturbances (Xiao *et al.,* 2020). The Himalayan region is the home of several essential stone species, most notably the *R. arboreum* Sm (Haq *et al.,* 2022). Recent past decades have

*Corresponding author e-mail: karthiyayini_bot@avinuty.ac.in

seen a growing interest in phytochemicals derived from plants (Chawla *et al.,* 2019), which are high in antioxidants, anti cancer potential and have a low toxicity to mammals. A large body of research suggests that antioxidant-rich foods containing phenolic compounds and other phytochemicals can reduce the effects of oxidative stress, which can lead to DNA mutations and cancer (Gautam *et al.*, 2021). As a result, researchers are increasingly interested in identifying and isolating plant antioxidants. Polyphenols and other compounds that have been proven antioxidants are abundant in plants, especially angiosperms (Hagerman *et al*., 1998). Polyphenols can scavenge free radicals and act as powerful antioxidants (Bennick, 2018).

Medicinal and nutritive plants have abundant uses and benefits. There has been a history of Indian Ayurvedic medicine and the immense diversity of its natural resources, which have made it possible to explore plants to treat many common ailments (Parveen *et al.,* 2020; Kumar *et al.,* 2020). The *Rhododendron* holds significant medicinal importance in Himalayan communities, with its flowers carrying multiple pharmacological significances (Tiwari *et al.,* 2020). *Rhododendron* plants offer a range of health benefits, including the treatment and prevention of heart diseases, dysentery, constipation, fever, detoxification, diarrhoea, inflammation, asthma, and bronchitis (Nisar *et al.*, 2013). In recent years, researchers have become increasingly interested in phytochemicals derived from plants that exhibit medicinal properties and possess high antioxidative, antimutagenic, and anticancer properties (Gautam *et al.*, 2020). In the present study, anticancer potential of methanolic extract of the plant was explored.

## MATERIALS AND METHODS

### Sample collection, preparation and extraction

Leaves and flowers of *R. arboreum* Sm was collected from Pedung village in West Kameng district of Arunachal Pradesh, India and authenticated at the BSI (Botanical Survey of India) Coimbatore. Tamil Nadu, No.: BSI/SRC/ 5/23/2021/Tech on 25/02/2021. The collected leaves and flowers were shade dried for two weeks and 20g of dried powdered leaves and flowers were sequentially extracted in 200 ml of methanol, ethanol, chloroform, acetone, petroleum ether and distilled water in each separate conical flask and incubated in incubation shaker at 150 rpm for 48 hours.

### Cell culture

In this study, human AGS cells were procured from the National Centre for Cell Sciences (NCCS), Pune, India. The cells were cultured in Dulbecco's modified eagle medium and 10% fetal bovine serum and centrifuged.

### Toxicity study-brine shrimp lethality assay

The *Rhododendron* flower methanol (RFM) and *Rhododendron* leaf methanol (RLM) powder extracts were measured and dissolved in distilled water to prepare a stock solution with a concentration of 1 mg/ml. The sample (RFM, RLM) of different volumes 100, 250, 500, 1000, 1500 µl were added to each beaker containing saline solution, respectively.

In accordance with standard procedures, the *Rhododendron* flower methanol (RFM) and *Rhododendron* leaf methanol (RLM) extracts were utilized in the brine shrimp lethality bioassay. Thirty shrimp specimens were introduced into solutions of various concentrations of the extracts. Shrimp movements were observed at intervals of 1, 2, 4, 6, and 24 h. The control groups included a blank solution with 30 shrimps in brine solution and a positive control using potassium dichromate (1 mg/ml). Shrimp mortality was assessed after 24 h. In each sample, 30 shrimps were introduced into 25 ml of the solution, and their mortality was monitored in comparison to the blank and positive control groups.

### *In vitro* cytotoxicity assay- An MTT assay for measuring mitochondrial synthesis

In standard cytotoxicity tests, cells undergo examination to assess their resilience against toxic substances. The assay is based on the principle that deceased cells or the byproducts of cell death do not reduce tetrazolium concentration, leading to variations that are notably dependent on cell number and mitochondrial activity (Kamiloglu *et al.,* 2020). The assay's efficacy relies on the ability of living cells to convert MTT into a blue formazan derivative, resulting in the formation of a distinctly blue-coloured product. This conversion is catalyzed by the mitochondrial enzyme succinate dehydrogenase by the MTT (Subavathy *et al.,* 2021). The observation included

assessing both the cell count and the quantity of formazan produced by the cells.

Dulbecco's modified eagle medium and 10% fetal bovine serum adjusted the cell count to 1.0 x 105 cells per ml in 96 well flat bottom microtiter plates after centrifugation. Each well of a 96 well flat microtiter plate was given 100 µl of diluted cell suspension, corresponding to approximately 10,000 cells per well. A suspension of 100 ìl of different test sample concentrations was prepared with the pellets after centrifugation of the cells for 24 h. Microscopic examinations and observations were conducted every 24 h over a 48-hour period, maintaining an atmosphere with 5% $CO_2$. The plates were kept in an incubator at 37ºC with a 5% $CO_2$ atmosphere for the entire 48 h duration. Before the addition of dimethyl sulfoxide (DMSO), the plates were shaken and further incubated at 37ºC with a 5% $CO_2$ atmosphere for a minimum of 2 h. Following the 48 h incubation, 20 µl of MTT solution at a concentration of 2 mg/ml was added to MEM-PR. To solubilize the formazan, 100 µl of DMSO was added, and the plates were gently shaken for 2 h. Absorbance at 540 nm was measured using microplate readers.

**Detection of cell death by annexin V/FITC- PI apoptosis staining by flow cytometry analysis**

The process of FITC Annexin V staining was employed to detect the loss of membrane integrity that typically occurs in the final stages of cell death, encompassing both apoptosis and necrosis. To distinguish between early apoptotic, late apoptotic, and necrotic cells, FITC Annexin V was commonly paired with a vital dye such as propidium iodide (PI). Live cells with intact membranes exclude PI, while dead and damaged cells with compromised membranes allowed PI to penetrate. This staining method facilitated the identification and differentiation of cells in various stages of the apoptotic process.

After trypsinizing and centrifuging cells for 10 minutes at 5000 rpm, the supernatant was discarded. 100 µl of 1X binding buffer was added to the pellet and it was shook vigorously. We stained the cells for 15 minutes at room temperature in dark with Annexin V/FITC and propidium iodide at room temperature. BD FACS verse flow cytometry was used to observe the cells after the incubation period. 400 µl of binding buffer was added and mixed well (Figure 3).

**Statistical analysis**

Linear regression was used to analyze the results of triplicate experiments, and results are presented as mean standard deviation (SD), % cell inhibition and log 10 concentration using Graph Pad prism software.

## RESULTS

**Brine shrimp lethality assay**

The results on the lethality of RFM and RLM extract on brine shrimps was recorded with $LC_{50}$ values. The sample

**Table 1:** Brine shrimp lethality assay

| S.No | Sample code | Concentration (µg/ml) | Mortality of Brine shrimp (No. of shrimps dead) (hrs) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 4 | 6 | 24 | % mortality (at 24 h) |
| 1. | RFM | 100 | 0 | 0 | 0 | 1 | 1 | 3 |
| | | 250 | 0 | 2 | 2 | 2 | 3 | 10 |
| | | 500 | 0 | 1 | 1 | 1 | 1 | 3 |
| | | 1000 | 0 | 0 | 2 | 2 | 2 | 7 |
| | | 1500 | 0 | 0 | 2 | 3 | 3 | 10 |
| 2. | RLM | 100 | 0 | 0 | 0 | 2 | 2 | 7 |
| | | 250 | 0 | 0 | 2 | 4 | 4 | 13 |
| | | 500 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 1000 | 0 | 0 | 1 | 2 | 2 | 7 |
| | | 1500 | 0 | 1 | 1 | 2 | 4 | 13 |
| 3. | Control $K_2Cr_2O_7$ | 1 (mg/ml) | 30 | - | - | - | - | 100 |
| 4. | Blank | Saline water | 0 | 0 | 0 | 0 | 0 | 0 |

(RFM, RLM) was comparatively less toxic than $K_2Cr_2O_7$ which showed maximum lethality of shrimps at higher concentration. The shrimps in the sample (RFM, RLM) were found to be very less toxic in lower concentration as well as in higher concentration. Even after 24 h, 1-4 shrimps were found to be mortal at highest concentration (Table 1).

**Incidence of lethality in brine shrimp**

Using brine shrimp lethality bioassay, regular and low concentrations of RFM and RLM were investigated in order to predict the toxicity of the chemicals on normal cells. This study found that RFM and RLM were less toxic for brine shrimps. As a result of comparing the concentrations of RFM and RLM to the mortality rate of brine shrimps, it was observed that mortality rates increased with the concentration of the test samples, which would made this substance a potential cytotoxic and toxic substance since its $LC_{50}$ is approximately 1000 g/ml. Therefore, the $LC_{50}$ value indicated that both the RFM and RLM extracts had lower toxicity effects on the brine shrimps. In accordance with the $LC_{50}$ values, both the RLM and RFM were considered as low toxicity agents for future experiments since they showed low toxicity against normal cells.

**Cancer cell growth inhibition activity**

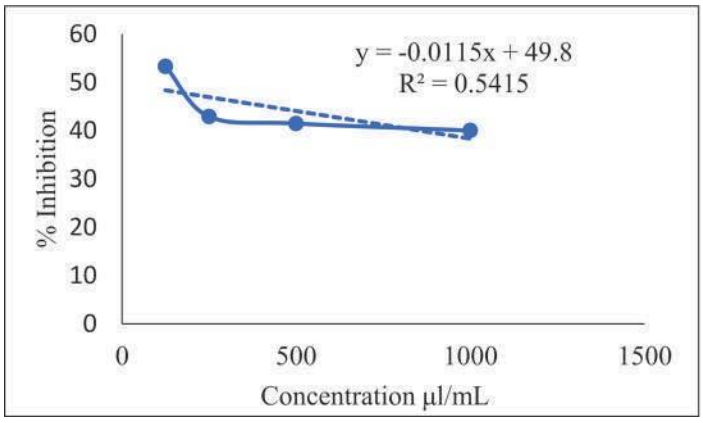
Cell viability was assessed using the MTT assay to evaluate the cytotoxic activity of *Rhododendron* flower methanol (RFM) and *Rhododendron* leaf methanol (RLM) extracts on AGS cells. The methanol extract of RLM exhibited the highest cytotoxic effect against AGS cell lines, with a % inhibition ranging from 43.5 ± 56.4% at the lowest concentration (125 µg/ml) to 66.2 ± 33.7% at the highest concentration (1000 µg/ml) (Figure 2). Following closely, RFM displayed cytotoxic effects ranging from 46.7 ± 53.2% at 125 µg/ml to 59.9 ± 40.0% at 1000 µg/ml on AGS cell lines (Figure 1). The analysis of cytotoxicity for the methanol extract of *Rhododendron* flowers and leaves revealed significant cytotoxic activity, with $IC_{50}$ values of 0.541 mg/ml and 0.841 mg/ml, respectively.

***In-vitro* cytotoxicity study**

The inhibitory effects of RFM and RLM extracts on the cancer cell line (AGS) were assessed *in vitro* using the MTT assay. This assay involved the conversion of water-soluble blue dyes into formazan through viable cells. Formazan, resulting from the reduction of the mitochondrial oxidoreductase enzyme, appeared as a purple-colored substance insoluble in water (Ivanova *et al.,* 2023). The amount of MTT-formazan produced during the dissolution of MTT-formazan in a chosen solvent was measured using a spectrophotometer. Cancer cell lines were initially seeded in culture flasks and allowed to grow to a sub-confluent state. Subsequently, RFM and RLM extracts were serially diluted in the growth medium of 96-well plates to create different concentrations (1000, 500, 250, and 125 µg/ml).

**Detection of cell death by annexin V/FITC- PI apoptosis staining by flow cytometer analysis**

To distinguish between apoptosis and necrosis of RFM and RLM, cells were harvested and stained with Annexin VFITC and PI. The combination of Annexin V and PI was widely used to assess the different stages of apoptosis in drug-



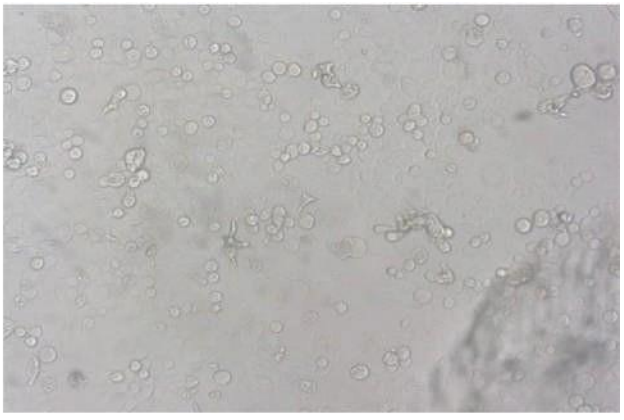



**Figure 1: Cytotoxicity of flower *R. arboreum* on AGS**

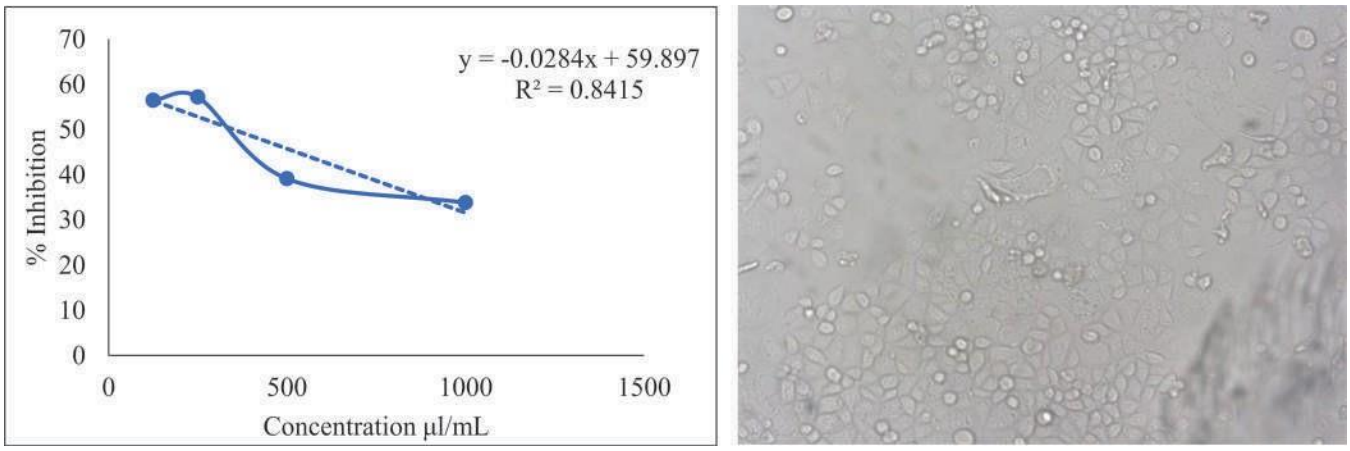



**Figure 2: Cytotoxicity of Leaf of *R. arboreum* on AGS**

treated cells (Manogaran *et al.,* 2023). The results showed that apoptosis was higher in the flower-treated cells than in the leaf (Figure 3). The cytotoxicity study of drugs was also correlated with result in the study.

**Evaluation of cell apoptosis**

Cellular suicide or self-destruction is defined as chromatic condensation, membrane swelling, cellular deformation, and shrinkage associated with apoptosis (Matthews *et al.,* 1997). In contrast to necrosis, apoptotic cells separate into several apoptotic bodies that are cleared by macrophages without causing inflammation. Apoptotic processes are mediated by proteolytic caspase proteins and till date, there have been about 14 caspases found in mammals. The apoptotic caspases can be divided into three groups: initiators, effectors and inflammators (Lavrik *et al.,* 2005). Our investigation of the apoptotic effect of RFM and RLM on AGS cells was conducted in order to understand how RFM and RLM plays an important role in controlling tumor growth. Under a microscope, we observed distorted nuclei and shrunken nuclei after treatment.

## DISCUSSION

The toxicity test results showed that RLM caused the highest mortality rate in the tested shrimps (13%) followed by RFM (10%). Based on the available literature data, there is a notable scarcity of research studies dedicated to investigating the *in vivo* toxicity of *R. arboreum*. Judzentiene *et al.* (2020) reported the results of *in vivo* toxicity tests performed with brine shrimp (*Artemia salina*) larvae, indicating diverse $LC_{50}$ average values ranging from 11.23 to 20.50 µg/ml. Marsh rosemary (*Rhododendron tomentosum* H) inflorescence oil (3 Fl) demonstrated lower toxicity, showing an $LC_{50}$ value of 20.50 µg/ml. A plant extract is deemed to have anti-cancer properties if its $LC_{50}$ value is below 20 µg/ml. We also monitored the number of deceased shrimps for each sample concentration after 24 h, and our results align with the findings reported by Judzentiene *et al.* (2020). In our findings, the lethality test results for RFM and RLM showed $LC_{50}$ values of 18.45 µg/ml and 13.83 µg/ml, respectively. These findings suggest the anti-cancer activity of the plant extracts, indicating their potential in anti-cancer research. The study provides insights into the toxicity levels of RFM and RLM and suggests their significance for further exploration in research and clinical applications.

In investigating the potential anti-cancer effects of RFM and RLM extracts on AGS cells, our study confirmed that the inhibitory impact on AGS cell proliferation was contingent upon the concentration of the drug and the initial cell density. Even at a concentration as high as 1000 µg/ml, the RLM extract exhibited significant inhibition, particularly against densely populated AGS cells. Following 24 and 48 h of incubation with RFM and RLM extracts, respectively, a substantial and significant inhibition of AGS cells was observed. These results suggest that RFM and RLM extracts may contain a diverse array of cytotoxic agents, indicating potential applications in the treatment of cancerous cells. Further research may reveal specific

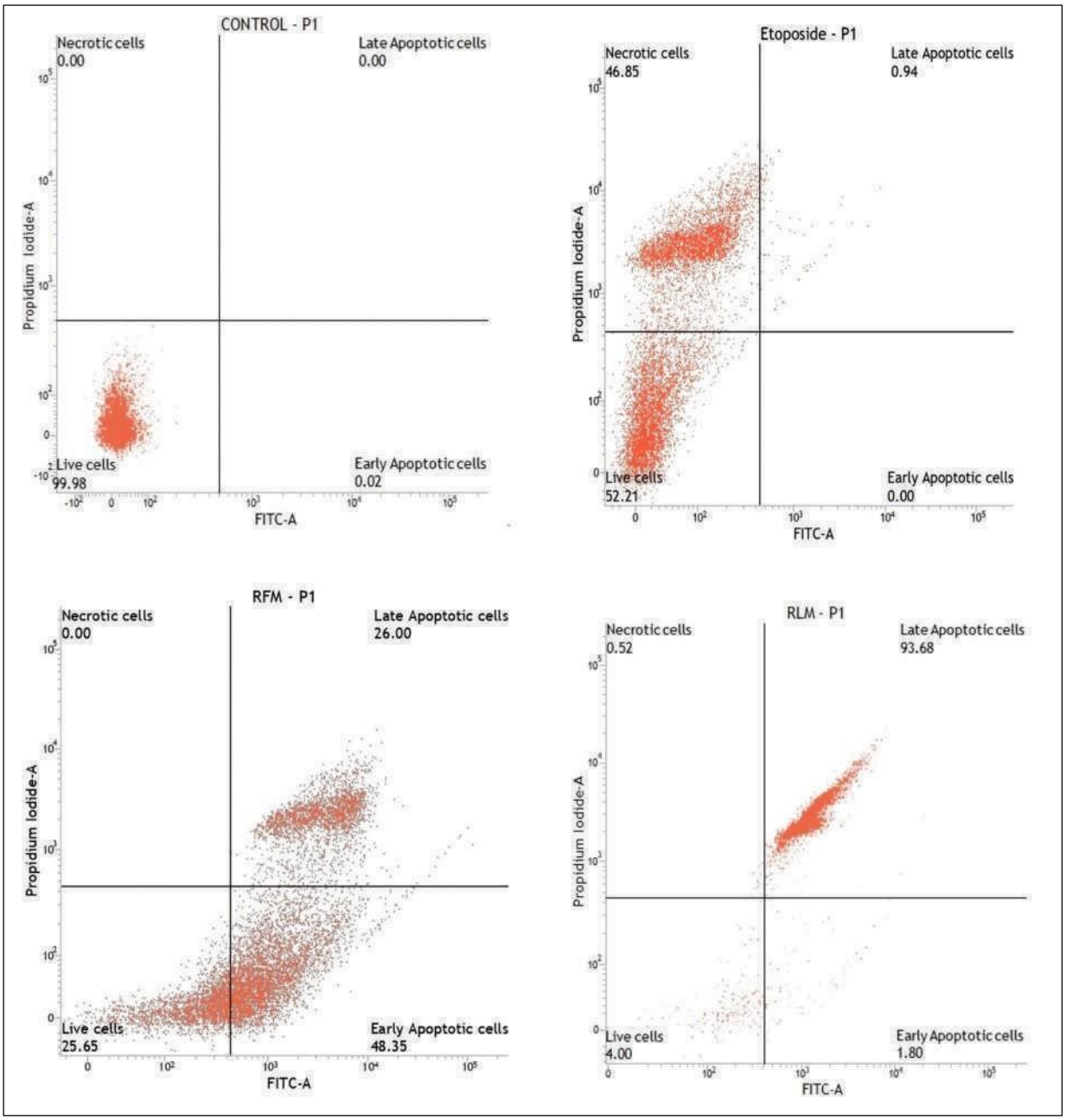



**Figure 3: Flow Cytometry analysis of *Rhododendron arboreum* Sm flower and leaf**

applications for the treatment of both cancer and normal cell populations based on these encouraging results.

A 48 h treatment with 1000 µg/ml was slightly toxic to normal cells for both RFM and RLM extracts, aligning with the acceptable toxicity for cancer cells 50% survival (Cota *et al.*, 2022). The RLM extract didn't harm the cells much, but at a concentration of 1000 µg/ml, it significantly slowed down their growth. Interestingly, even though this concentration was most effective against cancer cells, it also affected the survival of normal cells by reducing their viability. Examining the survival trend line at different concentrations, we observed a significant increase in the reduction of cancer cell survival with an increase in treatment concentration. Although this trend was somewhat

similar for normal cells, indicating a potential differential response between cancer and normal cells. Similarly, Bilir *et al*. (2018) found that the extract from *Rhododendron ponticum*, a genus of *Rhododendron*, caused harm or cell death in prostate cancer cells. They suggested this might be due to reduced mitochondrial activity, a result similar to what we observed in our study using the MTT assay to check cell viability. They also mentioned that *Rhododendron brachycarpum* had an anti-cancer effect on various human cancer cell lines, including A549, AGS, Hep3B, and MCF-7. However, it's important to consider these findings in future studies and clinical trials. In our study, the RFM and RLM extracts showed cytotoxic effects and could potentially lead to increased DNA fragmentation in AGS cells, triggering apoptosis.

In this investigation, Annexin V-FITC/PI staining was used to quantify apoptotic cell percentage. The results demonstrate a concentration-dependent increase in the rate of apoptosis induced by RFM and RLM extracts in AGS cells. This signifies the cytotoxic potential of these extracts through the promotion of apoptosis. As outlined by Ndozangue *et al.* (2008) apoptosis is characterized by distinct morphological alterations, including membrane swelling, cellular shrinkage, chromatin condensation, and the formation of apoptotic bodies. Additionally, the intricate role of mitochondria in apoptosis progression involves a decline in $\Delta\psi m$ (mitochondrial membrane potential), culminating in the disruption of the outer mitochondrial membrane, the opening of the permeability transition pore, and subsequent release of pro-apoptotic factors (Bouchier *et al*., 2005). Our observations in this study revealed decline in $\Delta\psi m$ with escalating concentrations of RFM and RLM extracts, providing compelling evidence for their apoptotic induction in AGS cells.

## CONCLUSION

The results of the study suggest that *Rhododendron* extract can be used to treat cancer patients by producing many secondary metabolites. The selected species have both preventive and therapeutic properties, which can be beneficial in treating cancer. Low toxicity on normal cells is demonstrated by both the RFM and RLM by the $LC_{50}$ value, which suggested future studies for better authentic application of the target products.

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### Conflict of interest

The authors declare no conflict of interest.

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# Phytochemical profiling and antioxidant evaluation of *Rhododendron arboreum* Sm leaf and flower: integrative analysis using advanced analytical techniques

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RESEARCH ARTICLE



# Phytochemical profiling and antioxidant evaluation of *Rhododendron arboreum* Sm leaf and flower: integrative analysis using advanced analytical techniques

Yangchen Dolma Kom, Karthiyayini Ramaswamy and Surya Suresh

Department of Botany, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, Tamilnadu, India

## ABSTRACT

**Objective:** This study investigates the biological activities of *Rhododendron arboreum* Sm from the eastern Himalayas, addressing a literature gap on its properties. It explores the plant's phytochemical, antioxidant, and medicinal characteristics.

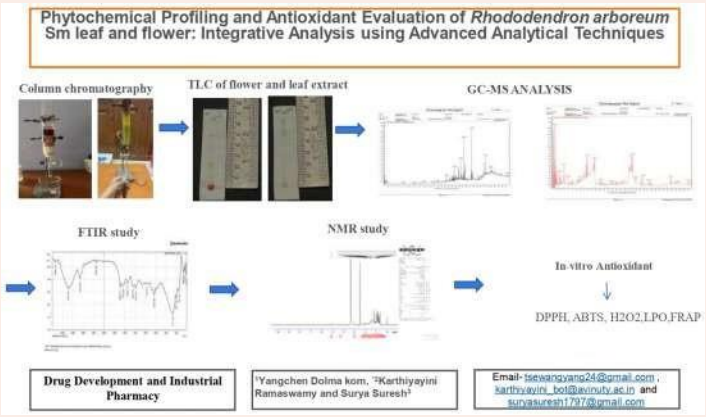
**Significance:** Evaluating methanolic extracts of *R. arboreum* offers valuable insights into its bioactive potential. Comprehensive GC-MS analysis identified a diverse array of compounds, highlighting the plant's chemical composition.

**Methods:** Methanolic leaf and flower extracts underwent sequential extraction and phytochemical profiling using column chromatography, TLC, and GC-MS analysis. Spectral studies aided compound identification, and antioxidant activity was assessed *via* spectrophotometric assays.

**Results:** Column chromatography separated methanol leaf and flower extracts into 17 and 24 distinct fractions, respectively. TLC analysis showed specific $R_f$ values for leaf (0.58, 0.65, 0.75, 0.8, 0.86, 0.9) and flower samples (0.91, 0.38, 0.48, 0.51, 0.56, 0.6, 0.65, 0.75, 0.85, 0.96). GC-MS analysis revealed a variety of organic functional groups, including aliphatic hydrocarbons, aromatic compounds, heterocyclic molecules, phenolic compounds, steroids, terpenoids, alcohols, esters, and other bioactive compounds. FTIR spectra identified functional groups such as hydroxyls, primary amines, alkanes, and alkynes. NMR data indicated a complex molecular composition with diverse proton environments. Leaf extracts demonstrated superior antioxidant activity compared to flower extracts in DPPH, ABTS, hydrogen peroxide scavenging, lipid peroxidation inhibition, and FRAP assays.

**Conclusion:** The study identifies diverse phytochemicals in *R.arboreum* extracts and highlights their potential applications in pharmaceuticals, nutraceuticals, and functional foods, owing to the superior antioxidant activity of leaf extracts compared to flowers.

## GRAPHICAL ABSTRACT

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## KEYWORDS

Column chromatography; TLC; GC-MS; biological activities; FTIR; NMR; *in vitro* antioxidant

## Introduction

*Rhododendron arboreum*, a plant from the Ericaceae family, is a traditional medicinal plant. Various parts of this plant are used to treat numerous ailments, particularly the leaves, which are employed to eliminate external parasites and to treat headaches, gout, and rheumatism [1]. To date, over 4,000 phytochemicals have been cataloged and classified based on their protective functions, as well as their physical and chemical characteristics. These phytochemicals are categorized as phenols, flavonoids, alkaloids, quinones, tannins, terpenes, glycosides, and polysaccharides, and they accumulate in various parts of plants, such as roots, stems, leaves, flowers, fruits, and seeds. The quantity and quality of phytochemicals in different parts of plants can vary depending on the

**CONTACT** Karthiyayini Ramaswamy Karthiyayini_bot@avinuty.ac.in Department of Botany, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, Tamilnadu, India

plant variety, processing methods, and environmental conditions [2]. Phytochemicals derived from plants, known for their high antioxidant, antimutagenic, and anticancer activities coupled with low toxicity to mammals, have garnered significant attention from researchers in recent decades. It is believed that oxidative stress and its consequences, such as DNA mutations and cancer development, can be mitigated by consuming antioxidant-rich foods containing phenolic compounds and other phytochemicals with antioxidant properties [3]. Phytocompounds such as taraxerol, ursolic acid acetate, betulinic acid, and leucopelargonidin have been identified in various extracts. The leaves of this tree are reported to contain glucoside, ericolin (arbutin), ursolic acid, α-amyrin, epifriedelinol, campanulin, quercetin, and hyperoside. Studies have demonstrated the presence of hyperoside (3-D-galactoside of quercetin), ursolic acid, and epifriedelinol [4]. The use of plant-derived natural compounds in therapeutic strate- gies has gained popularity due to their effectiveness and minimal adverse effects [5].

Recent advancements in analytical chemistry platforms, such as integrating mass spectrometry with techniques like gas chromatography (GC), liquid chromatography (LC), capillary electrophoresis (CE), and nuclear magnetic resonance (NMR) spectroscopy, have significantly enhanced the efficiency of metabolome analysis setups [6]. Chromatography and spectroscopy, highly effective and dependable tools for phytochemical analysis. Fourier transform infrared (FTIR) spectroscopy, specifically, is employed to characterize and identify functional groups with precision [7]. Examining spectral analyses of specimens from the same species enables assessment of variability in their chemical profiles. Consequently, FTIR spectroscopy emerges as a robust tool for analyzing variations in the proportions of major organic compounds not only across different species but also among specimens of the same species thriving in diverse environmental conditions [8].

The exploration of *R. arboreum* concerning phytochemical analysis, antioxidant studies, and spectral studies necessitates a thorough and systematic investigation into its chemical constituents and biological properties. While some studies have identified certain phytochemicals and evaluated antioxidant activities, there remains a lack of detailed investigation into the full spectrum of phytochemicals present in different parts of the plant (such as leaves and flowers) using advanced analytical techniques like GC-MS. Furthermore, there is a gap in understanding the specific spectral fingerprints (FTIR, NMR) of these phytochemicals, which are crucial for their structural identification and potential bioactivity correlation.

This study aims to explore *R. arboreum* using comprehensive GC-MS analysis to identify and characterize its chemical composition. Employing column chromatography, TLC, and spectroscopic methods, the research profiles phytochemicals and evaluates antioxidant activity. These insights are valuable for understanding the potential pharmaceutical and nutraceutical applications of *R. arboreum*.

## Materials and methods

### Collection and authentication

The leaves and flowers of *R. arboreum* Sm were collected in December 2020 from Pedung village of West Kameng district, Arunachal Pradesh, India. The specimens were authenticated and identified on 25/02/2021 at the Botanical Survey of India (BSI), Coimbatore, Tamil Nadu, under the reference number BSI/SRC/5/23/2021/Tech.

### Preparation of extracts

After shade-drying for two weeks, 20 g of dried, powdered leaves and flowers were mixed separately with 200 ml of various pure solvents of different polarity—ranging from nonpolar petroleum ether, chloroform, acetone, ethanol, and methanol to polar distilled water. Each mixture was incubated in a shaker at 150 rpm for 48 h at room temperature (25 °C–30 °C). Following incubation, the samples were macerated to separate the extracted plant material from the solvent-soaked residues, which were then preserved in Eppendorf tubes at room temperature (25 °C-30 °C) for further analysis.

### Qualitative analysis

Phytochemical screenings are essential preliminary tests designed to identify both primary and secondary metabolites present in plant extracts. These screenings employ various qualitative analyses, each tailored to detect specific classes of compounds. For alkaloids, tests such as Mayer's and Dragendorff's were utilized, while Molisch's and Benedict's tests were employed to detect carbohydrates. Glycosides can be identified using methods like the Saponin (Foam) Test, Legal's Test, and Borntrager's Test. Tests like Salkowski's and Liberman-Burchard's were used for detecting phytosterols, whereas flavonoids are identified using the Alkaline Reagent Test. Additionally, screenings cover other compounds such as acidic compounds, catechin, phlobatannins, volatile oils (using NaOH), reducing sugars (Benedict's Test), resins, starch (Iodine Solution Test), carotenoids, oxalates, and Vitamin C (DNPH test) [9–11]. These qualitative analyses collectively provide a comprehensive profile of the chemical constituents present in the plant extract Table 1.

### Quantitative analysis

In this study, absorbance measurements were conducted using a LABMAN LMSPUV1920 UV-Visible double beam spectrophotometer.

#### Quantification of flavonoids

Initially, a 0.5 ml aliquot of each sample was prepared and mixed with a reagent solution composed of 10% aluminum chloride, potassium acetate, and 80% methanol in a ratio of 1 ml per 0.5 ml of sample aliquot. This reagent mixture is known to form a stable complex with flavonoids. After thorough mixing, the samples were incubated at room temperature for a specified period to allow for color development. The absorbance of the resulting solutions was then measured spectrophotometrically at 415 nm. Each sample was analyzed in triplicate to ensure reproducibility, and results were expressed as mean ± standard deviation [12].

#### Quantification of total carbohydrate

Initially, a glucose stock solution and working standards were prepared with known concentrations ranging from 0 to 1 mg/mL. These solutions were mixed with a freshly prepared anthrone reagent, which reacts with carbohydrates to produce a green-colored complex. The mixtures were heated in a water bath and then allowed to cool to room temperature. The absorbance of the resulting green solutions was measured spectrophotometri- cally at 630 nm using a suitable spectrophotometer. Each sample was analyzed in triplicate to ensure precision, and results were reported as mean ± standard deviation [13].

### Protein estimation

Initially, samples and standards of known protein concentrations are prepared [14], the assay starts by mixing the sample extracts with the Lowry reagent, which typically contains copper ions and Folin-Ciocalteu reagent in an alkaline solution. This mixture leads to the formation of a complex between proteins and the reagents, resulting in a color change from green to blue. After incubation to ensure complete reaction, the absorbance of the blue-colored solution is measured spectrophotometrically at 660 nm. This wavelength is chosen for its sensitivity and reliability in detecting the presence of the protein-bound complex.

### Estimation of total tannin

Initially, tannins were extracted from *R. arboreum* using a solid-liquid extraction method. The plant material was ground into a fine powder and subjected to maceration with 80% methanol as the solvent. Once extracted, the tannin-containing extract is mixed with the Folin-Ciocalteau reagent, which contains phosphotungstic/phosphomolybdic acid complexes [15]. This mixture induces a chemical reaction where the tannins reduce the reagent, resulting in the formation of a blue-colored complex. The absorbance of this complex is measured spectrophotometrically at 725 nm, a wavelength chosen for its sensitivity to the blue color developed during the reaction. The absorbance readings of the sample are then compared against this calibration curve to quantify the total tannin content.

### Estimation of total alkaloids

Alkaloids were extracted from the sample using a solid-liquid extraction method [16]. The plant material was ground into a fine powder and subjected to maceration with 70% methanol as the solvent. Once extracted, the alkaloid-containing extract is mixed with a bromocresol green reagent solution. Bromocresol green forms a complex with alkaloids, resulting in a color change that correlates with the alkaloid concentration in the sample. The absorbance of the resulting colored solution is measured spectrophotometrically at a specific wavelength optimized for bromocresol green complex formation. Typically, the absorbance is measured at around 620 nm, as this wavelength is suitable for detecting the maximum absorbance of the bromocresol green-alkaloid complex.

### Estimation of total phenol

Samples were mixed with the Folin-Ciocalteau reagent, which contains phosphotungstic and phosphomolybdic acids. The reaction between the polyphenols and the Folin-Ciocalteau reagent leads to the formation of a blue-colored complex. This color change is indicative of the total polyphenol content present in the sample [17]. After allowing sufficient time for the color to develop, the absorbance of the resulting colored solution is measured spectrophotometrically, typically at a wavelength around 750 nm. This wavelength is chosen based on the absorbance maximum of the complex formed between polyphenols and the Folin-Ciocalteau reagent.

## Column chromatography

The methanol extract from *R. arboreum* leaves and flowers underwent column chromatography using a silica gel (60-120 mesh) glass column. Approximately 5 grams of the crude material were combined with 8 grams of silica gel and loaded onto a 46 × 2 cm column. The extract was eluted first with chloroform, followed by a gradient elution with chloroform: methanol of increasing polarity [18].

Subsequently, all collected fractions were analyzed using TLC (Thin Layer Chromatography) on silica gel 60 F254 plates, employing a Chloroform: methanol (95:5) solvent system for development. Yellow spots were detected on the plates. Fractions exhibiting similar $R_f$ values were consolidated, and the organic solvent was removed using a rotary evaporator.

## TLC – thin-layer chromatography

Plant extracts (6-20 µl) applied to silica gel 60 TLC plate. Chromatogram developed with mobile phase: ethyl acetate:methanol:formic acid:toluene (100:26:26:13 v/v/v/v) [19]. Plate removed at 50% development, solvent evaporated for visualization under visible light. Compounds identified using Rf values (retention factors) compared to known standards.

## GC-MS-gas chromatography-mass spectrometry

Agilent GC-MS (CH-GCMSMS02) used with 8890 GC and 7000 GC/TQ. Column: 30 m long, 250 µm diameter, 0.25-µm film. Helium mobile phase, nitrogen collision gas. Solvent: methanol. Data analyzed *via* Mass Hunter. Temperature: 50-120 °C at 5 °C/min (16 min), 120-210 °C at 10 °C/min (26 min), 210-280 °C at 10 °C/min (38 min). Scan range: 30-900 *m/z*. Concentration not estimated, qualitative analysis [20].

The crude samples were initially diluted with an appropriate solvent (1/100, v/v) and filtered to remove particles. Following preparation, 1 µL of the particle-free diluted crude extracts was injected using a syringe into the injector, with a split ratio of 30:1. Full-scan mass spectra were collected over a scan range of 40-550 atomic mass units (amu). The percentage composition of the crude extract constituents was determined based on peak area analysis. Identification and characterization of chemical compounds present in the crude extracts relied on matching GC retention times and mass spectra with those of standards available in mass spectrum libraries.

For interpretation *via* Gas Chromatography-Mass Spectrometry (GC-MS), the National Institute of Standards and Technology (NIST) database was utilized. Unknown component spectra were compared with spectra of known components stored in the NIST library, alongside retention time comparisons. This process enabled determination of the names, molecular weights, and structures of the components present in the test materials.

## FTIR - Fourier transform infrared spectroscopy

Based on how infrared light interacts with a substance's chemical composition, Fourier transform infrared spectroscopy (FTIR) is an extremely powerful analytical technique [21]. The dried powder extracted from various solvents of the leaf and flower sample was utilized for FTIR analysis, employing 100 mg KBr pellets as encapsulation in sample disks. The powdered plant material was loaded into an FTIR spectroscope (Shimadzu, IR Affinity 1, Japan), which scanned the spectral range from 400 to 4000 $cm^{-1}$ at a resolution of 4 $cm^{-1}$. This method facilitated the examination of the molecular composition and functional groups present in the plant extracts through infrared absorption spectroscopy.

### NMR - nuclear magnetic resonance

The extracts were prepared by transferring 700 µL of each sample into 5-mm NMR tubes for proton ($^1$H) NMR analysis. Using a Bruker AVANCE III instrument operating at 600.13 MHz and 25 °C, $^1$H NMR spectra were recorded with 16/64 scans, an acquisition time of 6.50 min per spectrum point, and a 5 s relaxation delay. Signal suppression of CD3OD and D2O solvent signals was achieved through selective low-power irradiation at $H_2O$ and CH3OH frequencies during the recycle delay. Free induction decays were Fourier transformed with LB = 0 Hz and spectra were processed for phase, baseline correction, and calibrated to 0.0 ppm using Topspin software (version 3.2, Bruker). Internal standards included 3-(trimethylsilyl)-1-propanesulfonic acid (TSP) and triiodobenzoic acid (TBA) [22].

Metabolite quantification using $^1$H NMR relies on the principle that signal intensity correlates with metabolite concentration. To quantify selected metabolites, the ratio method described was employed. An internal standard (TSP or TBA) with a known mass (mST) is utilized to calculate the concentration of the target metabolites using the following equation:

$$m_X = m_{ST} * (A_X / A_{ST}) * (MW_X / MW_{ST}) * (N_{ST} / N_X)$$

Here, $m_X$ represents the unknown mass of the target analyte, $A_X$ and $A_{ST}$ are the integral areas for the respective signals, $N_X$ and $N_{ST}$ denote the number of protons generating these signals, and $MW_X$ and $MW_{ST}$ are the molecular weights of the target analyte and the internal standard (TSP/TBA), respectively. This method enables precise determination of metabolite concentrations based on their $^1$H NMR spectra and the calibration provided by the internal standard.

### DPPH assay (2,2-diphenyl-1-picrylhydrazyl)

The antioxidant activity of *R. arboreum* leaf and flower extracts in methanol was assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) [23]. A DPPH solution was prepared in methanol and added to different concentrations of the extracts (50, 100, 150, 200, 250, and 300 ppm). The absorbance at 517 nm was measured to determine the extent of free radical scavenging activity. Ascorbic acid was employed as the standard antioxidant. These measurements were conducted in duplicate, and the percentage inhibition (Pi) was calculated using the following equation:

$$\mathbf{Pi = Ab - As \div Ab * 100\%}$$

The absorbance of the control (Ab) and the absorbance of the sample (As) were used to calculate the $IC_{50}$ values through linear regression analysis, which served to quantify the antioxidant capacity of the extract.

### ABTS assay (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid))

The stock solutions comprised a 7 mM ABTS solution and a 2.4 mM potassium persulfate solution. To create the working solution, equal volumes of these stock solutions were combined and allowed to react for 12 h at room temperature in darkness. Subsequently, the solution was diluted by mixing 1 ml of $ABTS^{\cdot+}$ solution with 60 ml of methanol to achieve an absorbance of 0.706 ± 0.001 units at 734 nm using a spectrophotometer. Fresh $ABTS^{\cdot+}$ solution was prepared for each assay [24].

For the assay, 1 ml of plant extract was mixed with 1 ml of the $ABTS^{\cdot+}$ solution, and the absorbance was measured at 734 nm after 7 min using a spectrophotometer. The $ABTS^{\cdot+}$ scavenging capacity of the extract was compared to that of ascorbic acid and the percentage of inhibition was calculated as:

$$\mathbf{Pi = Ab - As \div Ab * 100\%}$$

### $H_2O_2$- (hydrogen peroxide scavenging assay)

Separate tubes were prepared for each incubation period (5–60 min) containing $H_2O_2$ (0.7 mM, 160 µl) and phosphate buffer at pH 7 (84 mM, 350 µl). Leaves and flowers (0.1 U/ml, 40 µl) were added to each tube, and the mixture was incubated at 37 °C. After each incubation period, the absorbance at 560 nm was measured against a reagent blank consisting of phosphate buffer [25]. The decrease in color intensity indicates the scavenging of $H_2O_2$ by the plant material. Calculate the percentage inhibition of hydrogen peroxide using the formula:.

$$Pi = As - Ac \div Ac * 100$$

### Lipid peroxidation assay

A modified thiobarbituric acid reactive substances (TBARS) assay was utilized to quantify lipid peroxides using egg yolk homogenates as a lipid-rich medium, following the method outlined by Ruberto et al. [26]. Briefly, 0.5 ml of egg yolk homogenate (10% v/v) was mixed with 0.1 ml of the extract (10 µg/mL). The volume was adjusted to 1.0 ml with distilled water. Subsequently, 0.05 ml of $FeSO_4$ was added, and the mixture was incubated at 37 °C for 30 min. Then, 1.5 ml of acetic acid followed by 1.5 ml of TBA in SDS were added sequentially. The resulting mixture was vortexed and heated at 95 °C for 1 h. After cooling, 5 ml of butanol was added, and the mixture was centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was measured at 532 nm, and the percentage inhibition was calculated using the formula:

$$Pi = As - Ac \div Ac * 100$$

### FRAP assay (ferric reducing antioxidant power)

The total antioxidant activity (FRAP assay) of the extract was determined using a modified method based on Benzie and Strain [27]. The stock solutions included 300 mM acetate buffer (3.1 g $C_2H_3NaO_2 \cdot 3H_2O$ and 16 ml $C_2H_4O_2$), pH 3.6, a 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl, and a 20 mM $FeCl_3 \cdot 6H_2O$ solution. A fresh working solution was prepared by combining 25 ml of acetate buffer, 2.5 ml of TPTZ, and 2.5 ml of $FeCl_3 \cdot 6H_2O$, and the temperature was adjusted to 37 °C prior to use.

Plant extracts (150 µL) were allowed to react with 2850 µl of the FRAP solution for 30 min in the dark. The absorbance readings of the resulting ferrous tripyridyltriazine complex were measured at 593 nm. A standard curve was established as linear between 200 and 1000 µM $FeSO_4$. Results compared with those obtained using ascorbic acid as a reference.

$$\text{FRAP value} = As - \text{intercept} \div \text{slope}$$

### Statistical analysis

The analyses were performed in triplicate to ensure accuracy, and the results were presented as mean values accompanied by their standard deviations. Subsequently, $IC_{50}$ values for the antioxidant assays were calculated using the linear regression method. The experimental data underwent analysis using one-way factorial ANOVA, followed by the Tukey multiple range test at $\alpha = 0.05$.

## Results

### Qualitative phytochemical analysis

The phytochemical analysis revealed the presence of alkaloids, carbohydrates, saponins, phytosterols, flavonoids, proteins, amino acids, diterpenes, phenols and tannins, quinones, coumarins, acidic compounds, catechins, volatile compounds, resins, starch, carotenoids, oxalates, and vitamin C in the petroleum ether, chloroform, acetone, ethanol, methanol, and distilled water extracts of *R. arboreum* (Tables 2–3).

### Quantitative phytochemical analysis

This study evaluated the chemical composition of *R. arboreum* leaves and flowers using methanol, ethanol, and acetone as extraction solvents. The alkaloid content in leaves was found to be 48.61 ± 0.02% *Rhododendron* methanol leaf (RML), 47.84 ± 0.01% *Rhododendron* ethanol leaf (REL), and 75.95 ± 0.05% *Rhododendron* acetone leaf (RAL), while in flowers, it was 32.31 ± 0.03% *Rhododendron* methanol flower (RMF), 38.06 ± 0.04% *Rhododendron* ethanol flower (REF), and 34.71 ± 0.00% *Rhododendron* acetone flower (RAF) extracts respectively. For tannin content, leaves exhibited 40.62 ± 0.01% (RML), 28.75 ± 0.02% (REL), and 50.43 ± 0.02% (RAL), whereas flowers showed 51.57 ± 0.03% RMF, 80.01 ± 0.16% (REF) and 52.21 ± 0.08% (RAF). Carbohydrate content in leaves was 66.95 ± 0.12% (RML), 50.49 ± 0.04% (REL), and 73.62 ± 0.13% (RAL), and in flowers, it was 112.33 ± 0.07% (RMF), 111.25 ± 0.02% (REF), and 81.249 ± 0.01% (RAF). Protein content in leaves was 34.16 ± 0.01% (RML), 3.63 ± 0.03% (REL), and 29.07 ± 0.01% (RAL), while in flowers, it was 58.49 ± 0.01% (RMF), 79.14 ± 0.01% (REF), and 16.42 ± 0.04% (RAF) (Table 4).

The phenolic content was 40.5 ± 0.13% GAE/g (RML), 32.59 ± 0.05% GAE/g (REL), 34.5 ± 0.02% GAE/g (RAL), while the flower showed 41.92 ± 0.10% GAE/g (RMF), 47.16 ± 0.10% GAE/g (REF), 44.48 ± 0.13% GAE/g (RAF) Both the leaf and flower of *R. arboreum* exhibited similarly high total phenolic content ($p < 0.0001$), indicating no significant difference between them (Table 4).

The flavonoid content in leaves measured 397.1 ± 0.07% RE/g (RML), 547.7 ± 0.10% RE/g (REL), 391.63 ± 0.04% RE/g (RAL), while in flowers it was 398.0 ± 0.06% RE/g (RMF), 609.6 ± 0.15% RE/g (REF), 502.1 ± 0.11% RE/g (RAF) with the highest amount found in the flower extract of *R. arboreum* (Table 4). The ratio of flavonoids to total phenolic content was greater in flowers compared to leaves of the plants.

### Column chromatography

The methanol extracts obtained from dried *R. arboreum* leaves and flowers were processed through column chromatography using silica gel (60–120 mesh). This method separates compounds based on their differing polarities, allowing for the isolation of distinct fractions (Figure 1). In the case of the leaves, the chromatographic process produced 17 fractions, each characterized by specific $R_f$ values ranging from 0.06 to 0.93 (Table 5). These $R_f$ values indicate the relative mobility of the compounds on the chromatography plate, with higher values suggesting compounds that moved further up the plate with the solvent front. Some fractions, particularly those with multiple $R_f$ peaks, suggest complex mixtures of compounds.

For the flowers, 24 fractions were obtained, with predominant $R_f$ values clustering around 0.1, 0.76, 0.85, and 0.9 (Table 6). Fraction 12 and 13 did not yield detectable spots under the conditions tested, indicating a potential absence of compounds with significant UV absorption. Fraction 14 showed a prominent spot with an $R_f$ value of 0.56, suggesting a distinct compound present in this fraction. Similarly, fractions 19 and 21–24

**Table 1.** The extracts of leaves and flowers were used to analyze the presence of bioactive compounds.

| S. no | Test | Reaction Mixture | Observation |
|---|---|---|---|
| 1. | Alkaloids - Mayer's test | Plant extract + Mayer's reagent | Yellow precipitate formation indicates alkaloids |
| 2. | Alkaloids- Dragendorff's test | Plant extract + Dragendorff reagent | Brownish fluorescent precipitate formation |
| 3. | Carbohydrates - Molisch's test | Plant extract + Alcoholic α-napthol + Conc. Sulfuric acid | Violet color at interface indicates carbohydrates |
| 4. | Carbohydrates - Benedict's test | Plant extract + Benedict's reagent + Boiling | Orange-red precipitate indicates carbohydrates |
| 5. | Glycosides - Saponin (Foam) test | Diluted plant extract + Shaken in graduated cylinder | Formation of 1cm foam layer indicates saponins |
| 6. | Glycosides - Legal's test | Plant extract + Dil.HCl + Sodium nitroprusside | Pink to blood red color indicates cardiac glycosides |
| 7. | Glycosides - Borntragers test | Plant extract + Ferric chloride + Benzene + Ammonia | Rose pink color in ammonical layer indicates anthraquinone glycosides |
| 8. | Phytosterols - Salkowski's test | Plant extract + Chloroform + Conc. Sulfuric acid | Golden yellow color indicates phytosterols |
| 9. | Phytosterols - Liberman-Burchard's test | Plant extract + Chloroform + Conc. $H_2SO_4$ | Brown or red-colored ring on sulfuric acid layer indicates presence |
| 10. | Flavonoids - Alkaline Reagent test | Plant extract + Sodium | Intense yellow color turning colorless with sulfuric acid indicates flavonoids |
| 11. | Acidic Compounds | Plant extract + Sodium bicarbonate solution | Production of effervescence indicates acidic compounds |
| 12. | Catectin | Matchstick + Extract + Conc. HCl | Red or pink color in matchstick indicates presence |
| 13. | Phlobatanin | Aqueous extract + 1% aqueous HCl | Deposition of red precipitate indicates presence |
| 14. | Volatile Oils (NaOH) | Extract + Dil. Sodium hydroxide + Dil. HCL | White precipitate formation indicates presence |
| 15. | Reducing Sugar (Benedict's Test) | Extract + Benedict's reagent + Boiling | Yellow to orange color indicates presence |
| 16. | Resins | Various solvent extract + Acetic anhydride + Conc. $H_2SO_4$ | Orange to yellow coloration indicates presence |
| 17. | Starch (Iodine Solution test) | Extract + Iodine solution | Blue/black color change indicates presence |
| 18. | Carotenoids | Plant extract + 85% $H_2SO_4$ | Blue color at interface indicates the presence |
| 19. | Oxalate | Plant extract + Glacial acetic acid | Greenish-black color indicates the presence |
| 20. | Vitamin C (DNPH test) | Sample + Dinitrophenyl hydrazine + Conc. $H_2SO_4$ | Yellow precipitate formation indicates Vitamin C |

**Table 2.** Analysis of primary and secondary metabolites using qualitative methods for *R. arboreum*. Leaf.

| S.No | Test | Reagents | Petroleum Ether | Chloroform | Acetone | Ethanol | Methanol | Distilled Water |
|---|---|---|---|---|---|---|---|---|
| 1. | Alkaloids | Mayer's | + | + | + | + | ++ | ++ |
| 2. | Carbohydrate | Molisch's | − | + | + | ++ | ++ | + |
| 3. | Saponin | Distilled water | − | + | − | − | − | − |
| 4. | Phytosterol | Conc. $H_2SO_4$ | − | − | − | + | ++ | + |
| 5. | Flavonoid | Sodium hydroxide | − | − | − | + | ++ | + |
| 6. | Protein | Nitric acid | + | − | + | ++ | ++ | + |
| 7. | Amino acid | Ninhydrin | − | − | − | + | ++ | + |
| 8. | Diterpenes | Copper acetate | − | − | + | ++ | ++ | + |
| 9. | Phenol and tannin | 0.1% v/v $Fecl_3$ | − | − | + | + | + | − |
| 10. | Quinones | Conc $H_2SO_4$ + sodium hydrogen | − | − | − | + | + | − |
| 11. | Coumarins | Alcoholic sodium hydroxide | + | _ | + | + | ++ | − |
| 12. | Acidic compound | Sodium bicarbonate | + | − | + | + | + | − |
| 13. | Catectin | $Fecl_3$ | + | + | + | − | + | − |
| 14. | Volatile | Sodium hydroxide | + | − | − | − | + | − |
| 15. | Resin | Acetic anhydride | − | + | + | + | + | − |
| 16. | Starch | Iodine solution | − | + | + | + | + | − |
| 17. | Carotenoids | 85% $H_2SO_4$ | − | − | + | + | + | − |
| 18. | Oxalate | Glacial acetic acid | − | + | + | _ | + | − |
| 19. | Vitamin C | DNPH + Conc $H_2SO_4$ | + | + | + | + | + | + |

+ + High presence of respective class compound, + Presence of respective class compound and - Absence of respective class compound.

**Table 3.** Analysis of primary and secondary metabolites using qualitative methods for *R. arboreum* flower.

| S.No | Test | Reagents | Petroleum Ether | Chloroform | Acetone | Ethanol | Methanol | Distilled Water |
|---|---|---|---|---|---|---|---|---|
| 1. | Alkaloids | Mayer's | − | − | + | + | + | + |
| 2. | Carbohydrate | Molisch's | − | − | − | + | ++ | + |
| 3. | Saponin | Distilled water | − | − | − | − | − | ++ |
| 4. | Phytosterol | Conc. $H_2SO_4$ | + | + | + | ++ | ++ | + |
| 5. | Flavonoid | Sodium hydroxide | − | − | − | + | ++ | − |
| 6. | Protein | Nitric acid | − | − | − | ++ | ++ | + |
| 7. | Amino acid | Ninhydrin | + | − | + | + | + | − |
| 8. | Diterpenes | Copper acetate | − | − | ++ | ++ | ++ | + |
| 9. | Phenol and tannin | 0.1% v/v $Fecl_3$ | + | − | + | ++ | ++ | − |
| 10. | Quinones | Conc $H_2SO_4$ + sodium hydrogen | _ | + | + | ++ | ++ | − |
| 11. | Coumarins | Alcoholic sodium hydroxide | + | − | + | − | + | − |
| 12. | Acidic compound | Sodium bicarbonate | + | + | + | − | + | − |
| 13. | Catectin | $Fecl_3$ | + | − | ++ | + | ++ | − |
| 14. | Volatile | Sodium hydroxide | + | − | − | − | + | − |
| 15. | Resin | Acetic anhydride | + | − | + | + | + | + |
| 16. | Starch | Iodine solution | + | − | − | + | + | + |
| 17. | Carotenoids | 85% $H_2SO_4$ | _ | − | + | − | + | + |
| 18. | Oxalate | Glacial acetic acid | + | − | − | − | + | − |
| 19. | Vitamin C | DNPH + Conc $H_2SO_4$ | + | − | + | + | + | + |

+ + High presence of respective class compound, + Presence of respective class compound and - Absence of respective class compound.

**Table 4.** Analysis of primary and secondary metabolites using quantitative methods for *R. arboreum* leaf and flower.

| S.No | Plant parts | Solvent | Alkaloid Atropine/g | Tannin TAE/g | Carbohydrtae mg/g | Protein mg/g | Phenol GAE/g | Flavonoid RE/g |
|---|---|---|---|---|---|---|---|---|
| 1. | **Leaf** | Methanol | 48.61 ± 0.02% | 40.62 ± 0.01% | 66.95 ± 0.12% | 34.16 ± 0.01% | 40.5 ± 0.13% | 397.1 ± 0.07% |
| | | Ethanol | 47.84 ± 0.01% | 28.75 ± 0.02% | 50.49 ± 0.04% | 3.63 ± 0.03% | 32.59 ± 0.05% | 47.7 ± 0.10% |
| | | Acetone | 75.95 ± 0.05% | 50.43 ± 0.02% | 73.62 ± 0.13% | 29.07 ± 0.01% | 34.5 ± 0.02% | 391.63 ± 0.04% |
| 2. | **Flower** | Methanol | 32.31 ± 0.03% | 51.57 ± 0.03% | 112.33 ± 0.07% | 58.49 ± 0.01% | 41.92 ± 0.10 % | 398.0 ± 0.06 % |
| | | Ethanol | 38.06 ± 0.04% | 80.01 ± 0.16% | 111.25 ± 0.02% | 79.14 ± 0.01% | 47.16 ± 0.10% | 609.6 ± 0.15% |
| | | Acetone | 34.71 ± 0.00% | 52.21 ± 0.08% | 81.249 ± 0.01% | 16.42 ± 0.04% | 44.48 ± 0.13% | 502.1 ± 0.11% |

Values are expressed as mean ± SEM ($n = 3$).

exhibited specific $R_f$ values such as 0.46 and 0.91, indicating the presence of different compounds separated based on their mobility in the chromatographic system. The diverse chemical composition of *R. arboreum* leaf and flowers, which can be further analyzed to identify specific bioactive compounds of interest.

### *TLC - thin-layer chromatography*

Thin-layer chromatography (TLC) was conducted using pre-coated silica gel 60 F254 plates from MERCK, Darmstadt, Germany. Solvent systems of ethyl acetate:methanol:formic acid:toluene in a ratio of 100:26:26:13 (v/v/v/v). The formula for calculating the $R_f$ (retention factor) value in TLC is:.

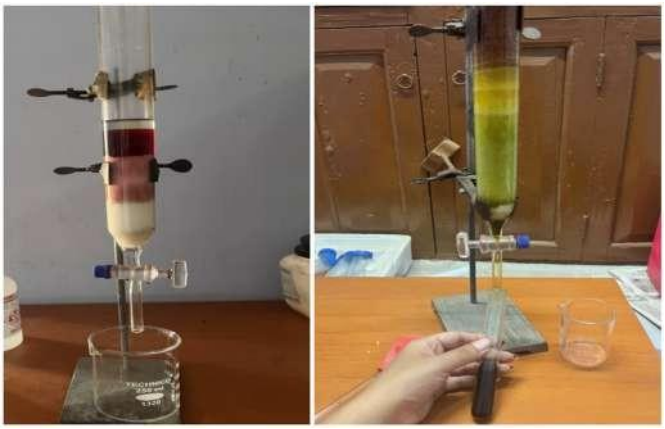

**Figure 1.** Column chromatography of *R. arboreum* flower and leaves extract.

**Table 5.** $R_f$ Values of leaf extraction fraction of *R. arboreum*.

| Fractions | $R_f$ values |
|---|---|
| 1-2 | 0.93 |
| 3 | 0.36, 0.88, 1.05 |
| 4 | 0.21, 0.66, 0.93 |
| 5 | 0.68, 0.93 |
| 6 | 0.35, 0.93 |
| 7 | 0.35, 0.73, 0.93 |
| 8 | 0.3, 0.75, 0.93 |
| 9 | 0.18, 0.93 |
| 10 | 0.15, 0.28, 0.51, 0.8, 0.93 |
| 11 | 0.16, 0.25, 0.55, 0.78, 0.93 |
| 12 | 0.16, 0.26, 0.55, 0.78, 0.9 |
| 13 | 0.15, 0.25, 0.5, 0.75, 0.9 |
| 14 | 0.08, 0.18, 0.26, 0.5, 0.75, 0.9 |
| 15 | 0.06, 0.16, 0.25, 0.5, 0.73, 0.93 |
| 16 | 0.1, 0.21, 0.26, 0.56, 0.93 |
| 17 | 0.06, 0.16, 0.55, 0.9 |

**Table 6.** $R_f$ Values of flower extraction fraction of *R. arboreum*.

| Fractions | Rf values |
|---|---|
| 1-3 | 0.76, 0.85, 0.9 |
| 4-8 | 0.1, 0.2, 0.76, 0.93 |
| 9-11 | 0.91 |
| 12-13 | – |
| 14 | 0.56 |
| 15 | – |
| 16 | 0.9 |
| 17 | 0.9 |
| 18 | – |
| 19 | 0.46 |
| 20 | – |
| 21-22 | 0.91 |
| 23 | 0.5, 0.91 |
| 24 | 0.91 |

$$R_f = \text{Distance travelled by component DC} \div \text{Distance travelled by solvents DS}$$

The obtained $R_f$ values for the flower samples are 0.91, 0.38, 0.48, 0.51, 0.56, 0.6, 0.65, 0.75, 0.85 and 0.96 (Figure 2). These values indicate different levels of mobility for the components in the flower sample, with the third component showing the highest mobility. For the leaf samples, the $R_f$ values are 0.58, 0.65, 0.75, 0.8, 0.86 and 0.9 (Figure 2). This suggests varying affinities for the stationary phase among the leaf components. The $R_f$ values offer a quantitative measure of component mobility during thin-layer chromatography. The observed differences in $R_f$ values between

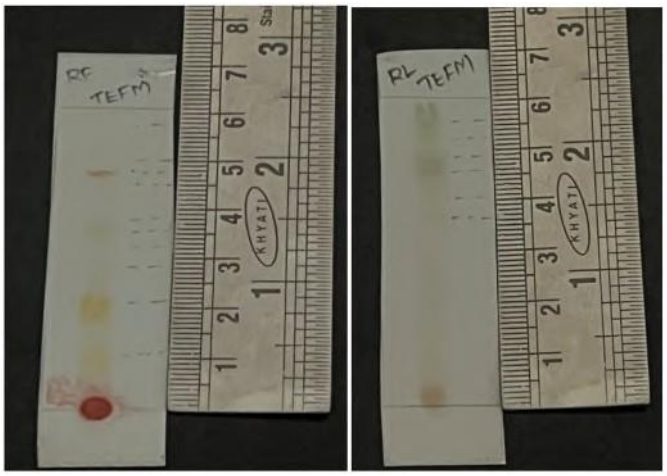

**Figure 2.** TLC of *R. arboreum* flower and leaf methanol.

flower and leaf samples indicate distinct chemical characteristics, contributing to a comprehensive understanding of the plant's composition.

## GC-MS - gas chromatography-mass spectrometry

In the crude extract from *R. arboreum* leaves and flowers, GC-MS analysis revealed the presence of aliphatic, aromatic, heterocyclic, phenolic, steroid, terpenoidal, alcohol, esters, and other functional groups that contain bioactive compounds. As a result of GC-MS chromatography, distinct peaks were identified for each component, each peak representing a specific compound in the sample. By analyzing retention times and mass spectra corresponding to these peaks, the compounds were identified. GC-MS analysis identified 20 compounds in the leaf sample of *R. arboreum* (Figure 3). These compounds include glycerin; silane; p-Mentha-1(7),2-dien-8-ol; 2,4-dithiapentane; L-alpha-terpineol; carvenone; 2,3-diketo-6-methoxybenzo[b]pyran; phenol; propionic acid; undecane; acetic acid; 2,4-di-tert-butylphenol; 2(4H)-benzofuranone; 1-hexadecanol; cyclohexane; neophytadiene; phytol; eicosanoic acid; hexadecanoic acid and vitamin E (Table 7).

Similarly, GC-MS analysis identified 26 compounds for the flower Sample of *R.arboreum* (Figure 4) and the compounds are Methylenecyclopropanecarboxylic acid; 4-Cyclopentene-1,3-dione; Methyl thiolacetate; 2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one; 2H-Pyran-2,6(3H)-dione; 2-Cyclopenten-1-one, 2-hydroxy-3-methyl; Furaneol; 3-Acetoxy-3-hydroxypropionic acid; 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl; Benzofuran, 2,3-dihydro; 5-Hydroxymethylfurfural; Glycerin; beta-Methyl xyloside; 1,2,3-Benzenetriol; 3-Furanacetic acid, 4-hexyl-2,5-dihydro-2,5-dioxo; Ethanol, 2,2′-oxybis-, diacetate; Phthalic acid, 2-isopropylphenyl methyl ester; beta-l-Arabinopyranoside, methyl; n-Hexadecanoic acid; 9,12-Octadecadienoic acid (Z,Z)-, methyl ester; 4-Imidazolidinecarboxylic acid, 4-hydroxy-2,5-dioxo; Hexadecanoic acid, 10-hydroxy-, methyl ester; Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester; gamma.-Sitosterol; 5H-Oxazolo[3,2-a] pyridine-8-carbonitrile, 6-ethyl-2,3-dihydro-2,7-dimethyl-5-oxo and alpha.-Amyrin (Table 8).

## FTIR - Fourier transform infrared spectroscopy

FTIR spectroscopic analysis detects various functional groups in bioactive compounds found in R. *arboreum* leaf and flower extracts. The extracts were analyzed within the FTIR spectrum, where

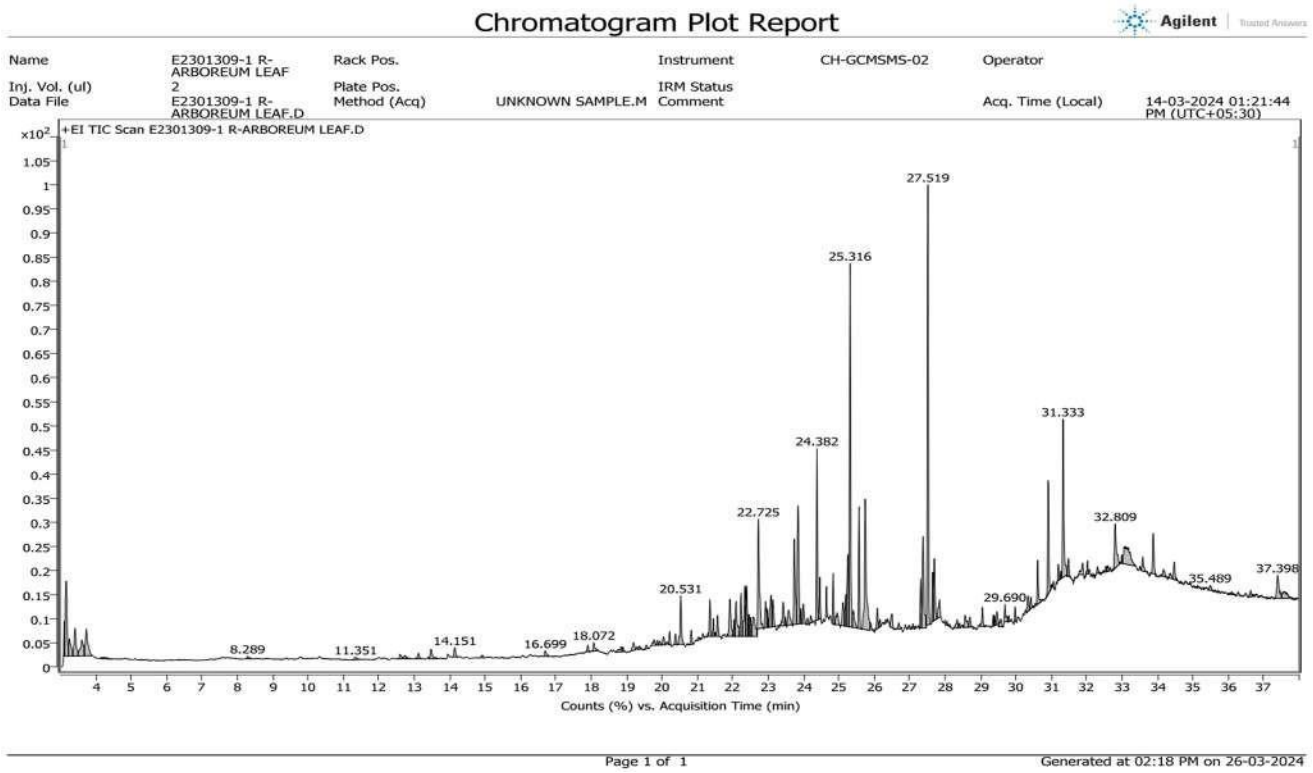


**Figure 3.** GC-MS chromatograms of *R. arboreum* Sm. leaf.

**Table 7.** List of phytochemicals identified in GCMS analysis of methanolic leaf extract of *R. arboreum* Sm.

| Compound Name | Molecular formula | Retention value (min) | Peak area (%) | Molecular weight (g/mol) | Biological activities | Reference |
|---|---|---|---|---|---|---|
| Glycerin | $C_3H_8O_3$ | 3.7337 | 14.77 | 92.09 | Anti-microbial | [43] |
| Silane | $H_4Si$ | 5.1076 | 1.81 | 32.116 | Dental Composite Development | [44] |
| P-Mentha-1(7),2-Dien-8-Ol | $C_{10}H_{16}O$ | 9.3850 | 1.41 | 152.23 | Biotransformations (Flavours, fragrances and Pharmaceuticals) | [45] |
| 2,4-Dithiapentane | $C_3H_8S_2$ | 10.3903 | 1.38 | 108.23 | Odorant | [46] |
| L-Alpha-Terpineol | $C_{10}H_{18}O$ | 12.6200 | 1.87 | 154.25 | Produces Vasorelaxation | [47] |
| Carvenone | $C_{10}H_{16}O$ | 14.1790 | 3.35 | 152.23 | Efficacious treatment to reduce both the intensity and frequency of migraine attacks. | [48] |
| 2,3-Diketo-6-Methoxybenzo[B] Pyran | $C_{10}H_8O_4$ | 14.9253 | 1.07 | 192.17 | Conversion of Cellulose to Bio-Oil in Hot-Compressed water with Ultrasonic Pretreatment | [49] |
| Phenol | $C_6H_6O$ | 16.6933 | 2.47 | 94.11 | Anti-Gastric Cancer | [50] |
| Propionic Acid | $C_3H_6O_2$ | 17.3442 | 1.69 | 74.08 | Inhibits Gastric Cancer | [51] |
| Undecane | $C_{11}H_{24}$ | 18.0712 | 3.18 | 156.31 | Anti-Allergic and Anti-Inflammatory effects | [52] |
| Acetic Acid | $C_2H_4O_2$ | 19.2001 | 3.30 | 60.05 | Tumor necrosis was caused by acetic acid, either mucosal or serosal. | [53] |
| 2,4-Di-Tert-Butylphenol | $C_{14}H_{22}O$ | 20.2176 | 3.06 | 206.32 | Antioxidant | [54] |
| 2(4H)-Benzofuranone | $C_8H_6O_2$ | 20.5320 | 14.36 | 134.13 | Post Fermentation Fragrance | [55] |
| 1-Hexadecanol | $C_{16}H_{34}O$ | 21.3548 | 8.47 | 242.44 | Anti-Cancer | [56] |
| Cyclohexane | $C_6H_{12}$ | 23.1392 | 7.67 | 84.16 | Anti-Cancer | [57] |
| Neophytadiene | $C_{20}H_{38}$ | 24.3800 | 34.08 | 278.5 | Anti-Cancer | [58] |
| Phytol | $C_{20}H_{40}O$ | 27.5177 | 29.27 | 296.5 | Anti-Cancer | [32] |
| Eicosanoic Acid | $C_{20}H_{40}O_2$ | 29.6901 | 4.66 | 312.5 | Anti-Mutagenic | [59] |
| Hexadecanoic Acid | $C_{16}H_{32}O_2$ | 31.3358 | 42.27 | 256.42 | Anti- Gastric Cancer | [60] |
| Vitamin E | $C_2H_6O$ | 37.3844 | 11.05 | 46.07 | Reduced Risk of death due to Gastric Cancer | [61] |

distinct functional groups were identified based on their bonding positions. The resulting peaks conclusively confirm a diverse array of functional groups present in these bioactive compounds.

In the leaf extract, significant absorption peaks were identified at various wavelengths: 3880.78cm$^{-1}$ (O-H stretching, phenols), 3387.00 cm$^{-1}$ (N-H stretching), 2924.09 cm$^{-1}$ (C-H stretching, aliphatic hydrocarbons), 2306.86 cm$^{-1}$ (C≡N stretching), 1689.64 cm$^{-1}$ (C=O stretching, ketones/aldehydes), 1627.92 cm$^{-1}$ (C=C stretching, alkenes), 1550.77 cm$^{-1}$ (N-H bending), 1442.75 cm$^{-1}$ (C-H bending), 1381.03 cm$^{-1}$ (C-N stretching), 1172.72cm$^{-1}$ (C-O stretching, ethers), 1033.85cm$^{-1}$

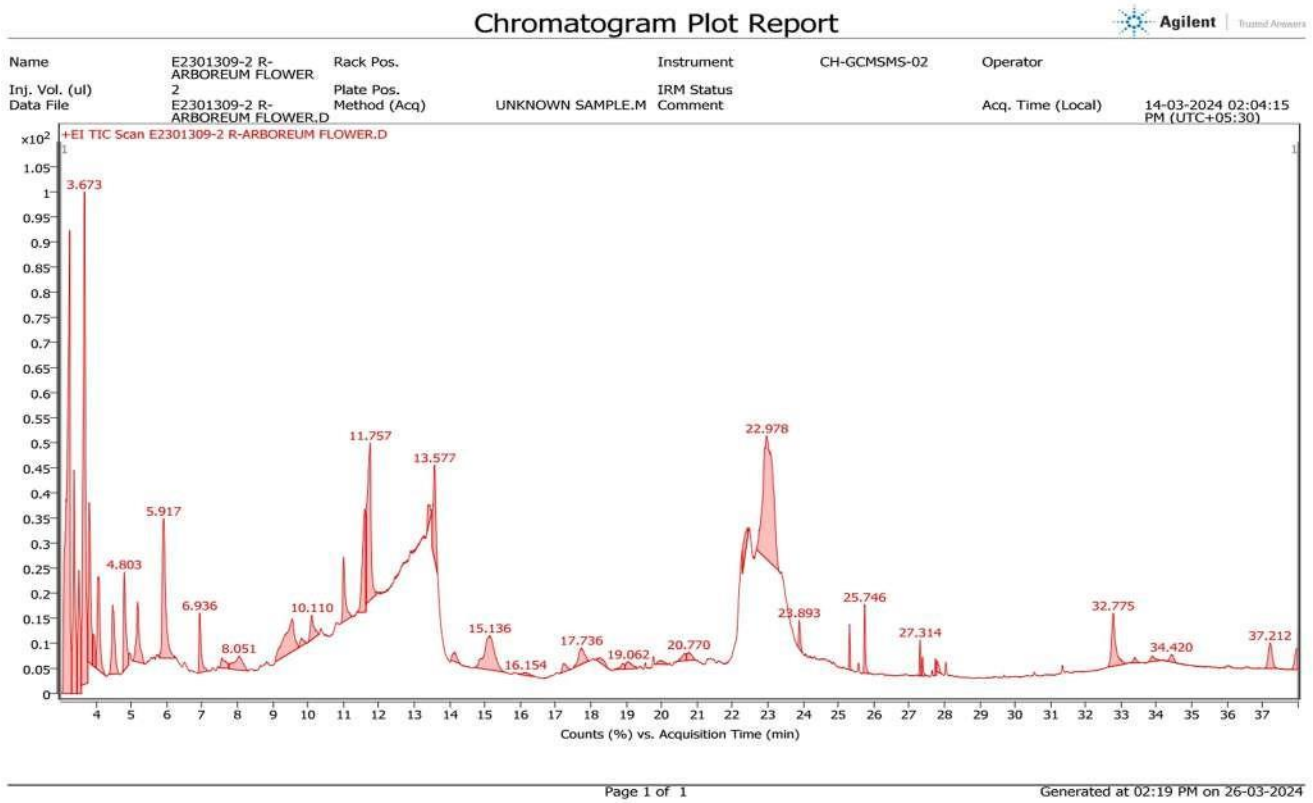


**Figure 4.** GC-MS chromatograms of *R. arboreum* Sm. flower.

(C-O-H bending, alcohols), 671.23 $cm^{-1}$ (C-Br stretching), 601.79 $cm^{-1}$ (C-Cl stretching), 547.78 $cm^{-1}$ (C-N stretching, amines), 462.92 $cm^{-1}$ (C-N-H bending), and 439.77 $cm^{-1}$ (C-S-H stretching) (Figure 5). Similarly, in the flower extract, distinct peaks were observed at 3718.76 $cm^{-1}$ (O-H stretching, phenols), 3286.70 $cm^{-1}$ (N-H stretching), 2924.09 $cm^{-1}$ (C-H stretching, aliphatic hydrocarbons), 2314.58 $cm^{-1}$ (C≡N stretching), 1689.64 $cm^{-1}$ (C=O stretching, ketones/aldehydes), 1604.77 $cm^{-1}$ (C=C stretching, alkenes), 1527.62 $cm^{-1}$ (N-H bending), 1442.75 $cm^{-1}$ (C-H bending), 1381.03 $cm^{-1}$ (C-N stretching), 1033.85 $cm^{-1}$ (C-O-H bending, alcohols), 671.23 $cm^{-1}$ (C-Br stretching), 594.08 $cm^{-1}$ (C-Cl stretching), 555.50 $cm^{-1}$ (C-N stretching, amines), 470.63 $cm^{-1}$ (C-N-H bending), and 416.62 $cm^{-1}$ (C-S-H stretching) (Figure 6).

## NMR nuclear magnetic resonance

### R. arboreum leaf NMR analysis

The NMR data reveals a variety of chemical shifts and integrals, indicating the presence of multiple proton environments in the molecule. Peaks in the downfield region (5.193–5.002 ppm) suggest the presence of deshielded protons, likely vinylic or aromatic. Peaks between 4.403–2.068 ppm indicate protons adjacent to electronegative atoms or carbonyl groups, such as -OCH or -CH2 groups. The majority of the peaks, from 1.963–0.630 ppm, correspond to aliphatic protons, suggesting a significant presence of $-CH_3$, $-CH_2$, and -CH groups. Notably, the large integrals around 1.587–1.383, 1.333–1.184, and 0.947–0.840 ppm indicate multiple similar proton environments, likely representing large groups of aliphatic protons. These patterns suggest that the molecule is likely a long-chain aliphatic compound, possibly with functional groups such as ethers, esters, or alcohols, characterized by multiple methyl and methylene groups. To determine the exact structure, additional information such as coupling constants, 2D NMR, or mass spectrometry data would be necessary (Figure 7).

### R. arboreum flower NMR analysis

The NMR data exhibits a diverse range of chemical shifts and integrals, indicating a complex molecular structure with multiple proton environments. The peaks in the downfield region (6.674–6.171 ppm) suggest the presence of highly deshielded pro- tons, likely part of aromatic or vinylic systems, with integrals indi- cating varying numbers of protons. The region between 5.436–4.172 ppm shows significant integrals, likely indicating pro- tons adjacent to electronegative atoms, such as -OCH groups, or protons within conjugated systems. The substantial integrals around 3.714–2.951 ppm point to a considerable number of protons, possibly from multiple methylene groups or complex functionalized groups.

The smaller peaks at 2.940–2.075 ppm correspond to protons near electron-withdrawing groups or part of functional groups such as carbonyls or alkenes. The region from 1.576–0.631 ppm, showing large integrals, indicates a significant presence of aliphatic protons, suggesting multiple methyl and methylene groups. Particularly, the large integral at 1.337–1.174 ppm and the broad region from 0.961–0.631 ppm likely represent substantial portions of aliphatic chains.

The molecule could be an aromatic compound with extensive aliphatic chains and several functional groups, such as ethers, esters, or alcohols, characterized by multiple methyl, methylene, and methine groups. Further detailed analysis, including coupling constants or additional 2D NMR data, would be essential for accurate structural elucidation (Figure 8).

**Table 8.** List of phytochemicals identified in GC-MS analysis of methanolic flower extract of *R. arboreum* Sm.

| Compound Name | Molecular formula | Retention value (min) | Peak area (%) | Molecular weight (g/mol) | Biological activities | References |
|---|---|---|---|---|---|---|
| 2-chloropropanamide | $C_3H_6ClNO$ | 3.1084 | 26.32 | 107.54 | Electrophiles in drug discovery. | [62] |
| Methylenecyclopropanecarboxylic acid | $C_5H_6O_2$ | 4.8031 | 34.74 | 98.1 | Anti-viral | [63] |
| 4-Cyclopentene-1,3-dione | $C_5H_4O_2$ | 5.1852 | 10.29 | 96.08 | Anti-fungal | [64] |
| Methyl thiolacetate | $C_3H_6OS$ | 6.4998 | 7.01 | 90.15 | Colorectal | [65] |
| 2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one | $C_6H_8O_4$ | 6.9349 | 26.3 | 144.12 | Anti-microbial | [66] |
| 2H-Pyran-2,6(3H)-dione | $C_5H_4O_3$ | 7.5665 | 8.03 | 112.08 | Anti-allergic | [67] |
| 2-Cyclopenten-1-one, 2-hydroxy-3-methyl | $C_6H_8O_2$ | 8.3932 | 1.53 | 112.13 | Anti-mycobacterial | [68] |
| Furaneol | $C_6H_8O_3$ | 9.5683 | 6.11 | 128.13 | Fermentation of wine | [69] |
| 3-Acetoxy-3-hydroxypropionic acid | $C_6H_{10}O_5$ | 11.0029 | 4.72 | 162.14 | Anti-bacterial | [70] |
| 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl | $C_6H_8O_4$ | 11.7562 | 5.19 | 144.12 | Antioxidant | [71] |
| Benzofuran, 2,3-dihydro | $C_{12}H_{15}NO_3$ | 13.5781 | 4.12 | 221.25 | Anti-cancer | [72] |
| 5-Hydroxymethylfurfural | $C_6H_6O_3$ | 14.1709 | 10.83 | 126.11 | Anti-gastric cancer | [73] |
| Glycerin | $C_3H_8O_3$ | 15.1407 | 2.91 | 92.09 | Anti-gastric cancer | [53] |
| beta-Methyl xyloside | $C_6H_{12}O_5$ | 17.7454 | 1.09 | 164.16 | Production of Xylanase | [74] |
| 1,2,3-Benzenetriol | $C_6H_6O_3$ | 19.0751 | 2.74 | 126.11 | Colorectal cancer | [75] |
| 3-Furanacetic acid, 4-hexyl-2,5-dihydro-2,5-dioxo | $C_{12}H_{16}O_5$ | 19.7737 | 7.62 | 240.25 | Anti-fungal and anti-microbial | [76] |
| Ethanol, 2,2′-oxybis-, diacetate | $C_8H_{14}O_5$ | 20.6499 | 13.97 | 190.19 | Antioxidant | [77] |
| Phthalic acid, 2-isopropylphenyl methyl ester | $C_{18}H_{18}O_4$ | 22.1377 | 1.15 | 298.3 | Anti-inflammatory and anti-cancer | [78] |
| Beta-L-Arabinopyranoside, methyl | $C_6H_{12}O_5$ | 22.9610 | 5.68 | 164.16 | Microbial biotransformation | [79] |
| n-Hexadecanoic acid | $C_{16}H_{32}O_2$ | 25.7483 | 7.94 | 256.42 | Antioxidant and anti-cancer | [80] |
| 9,12-Octadecadienoic acid (Z,Z)-, methyl ester | $C_{19}H_{34}O_2$ | 27.3131 | 2.22 | 294.5 | Anti-cancer | [81] |
| 4-Imidazolidinecarboxylic acid, 4-hydroxy-2,5-dioxo | $C_4H_4N_2O_5$ | 29.2983 | 43.2 | 160.09 | Not Reported | |
| Hexadecanoic acid, 10-hydroxy-, methyl ester | $C_{17}H_{34}O_3$ | 30.5427 | 100 | 286.4 | Anti-bacterial | [82] |
| Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester | $C_{19}H_{38}O_4$ | 31.3363 | 2.03 | 330.5 | Anti-ulcer | [83] |
| gamma.-Sitosterol | $C_{29}H_{50}O$ | 32.7738 | 4.89 | 414.7 | Antioxidant, anti-bacterial and anti-cancer | [84] |
| 5H-Oxazolo[3,2-a]pyridine-8-carbonitrile, 6-ethyl-2,3-dihydro-2,7-dimethyl-5-oxo | $C_{12}H_{14}N_2O_2$ | 33.3953 | 2.27 | 218.25 | Nephroprotective | [85] |
| alpha.-Amyrin | $C_{30}H_{50}O$ | 34.4448 | 1.25 | 426.7 | Anti-cancer | [86] |

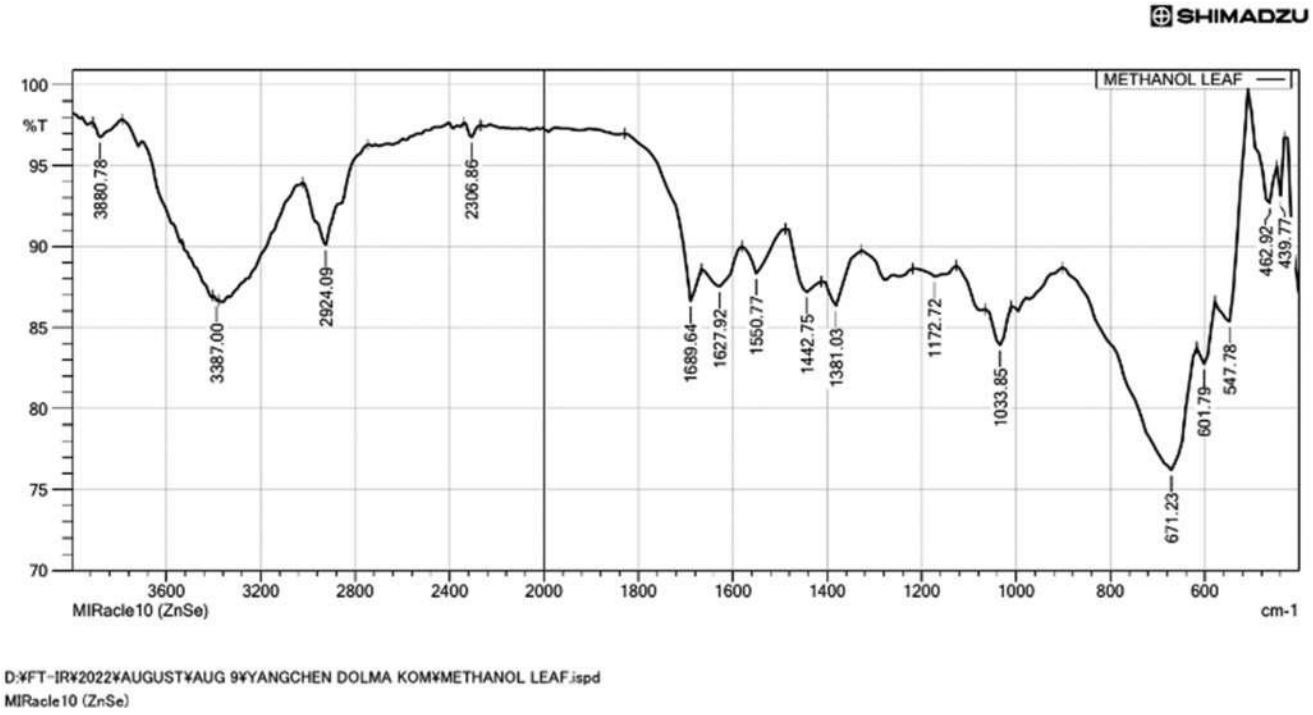


**Figure 5.** FTIR of *R. arboreum* Sm methanol extracts of leaf.

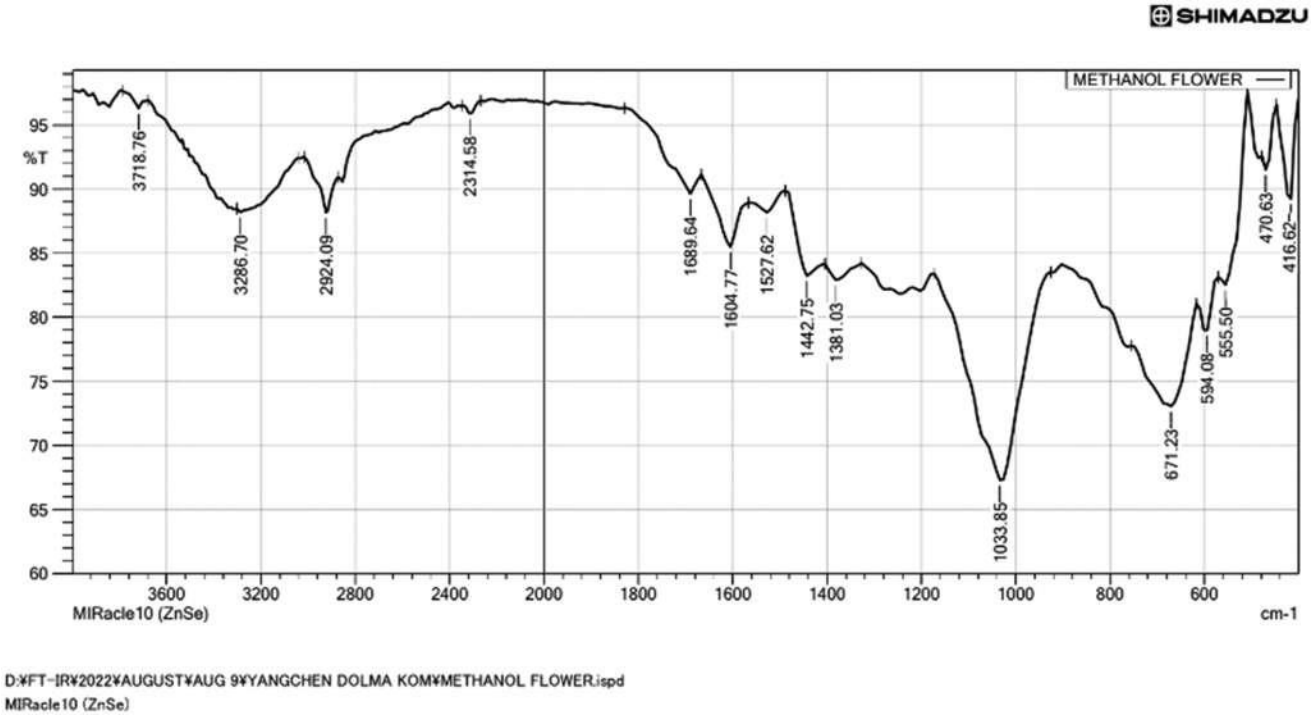


**Figure 6.** FTIR of *R. arboreum* methanol extract of flower.

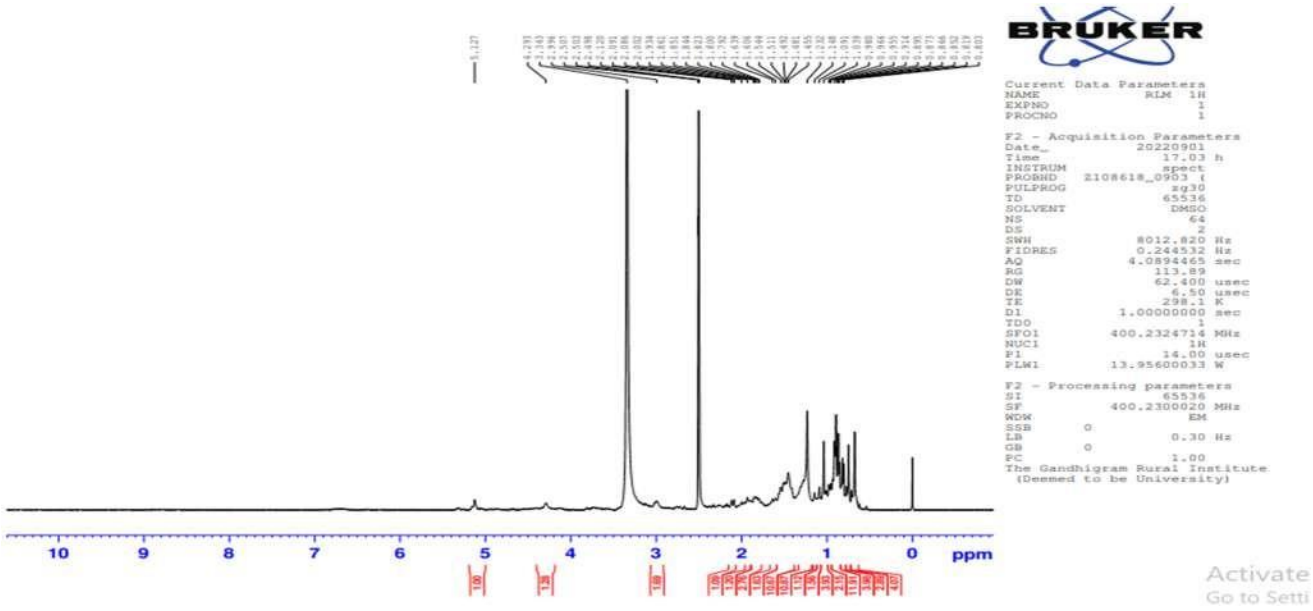


**Figure 7.** NMR analysis of *R. arboreum* methanol leaf extract.

### *DPPH radical scavenging activity*

The antioxidant potential of methanol extracts from leaves and flowers of *R. arboreum* was evaluated using the DPPH scavenging assay. Both extracts exhibited substantial antioxidant activity, demonstrating effectiveness comparable to the reference standard antioxidant, ascorbic acid. The DPPH assay relies on the ability of DPPH, a stable free radical, to change color (from purple to yellow) upon reduction by antioxidants. The radical's absorbance at 517 nm decreases with the transfer of an electron from an antioxidant to the DPPH radical. The methanol extracts from both leaves and flowers of *R. arboreum* showed significant inhibition of DPPH activity. Specifically, the $IC_{50}$ values for the methanol extracts of *R. arboreum* leaves and flowers were determined to be 56 µg/mL and 67 µg/mL, respectively, indicating their potent antioxidant capabilities (Figure 9).

### *ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radicals*

The ABTS cation assay was employed to evaluate the scavenging activity of methanol extracts from both the leaves and flowers of *R. arboreum*. The assessment covered a concentration range of

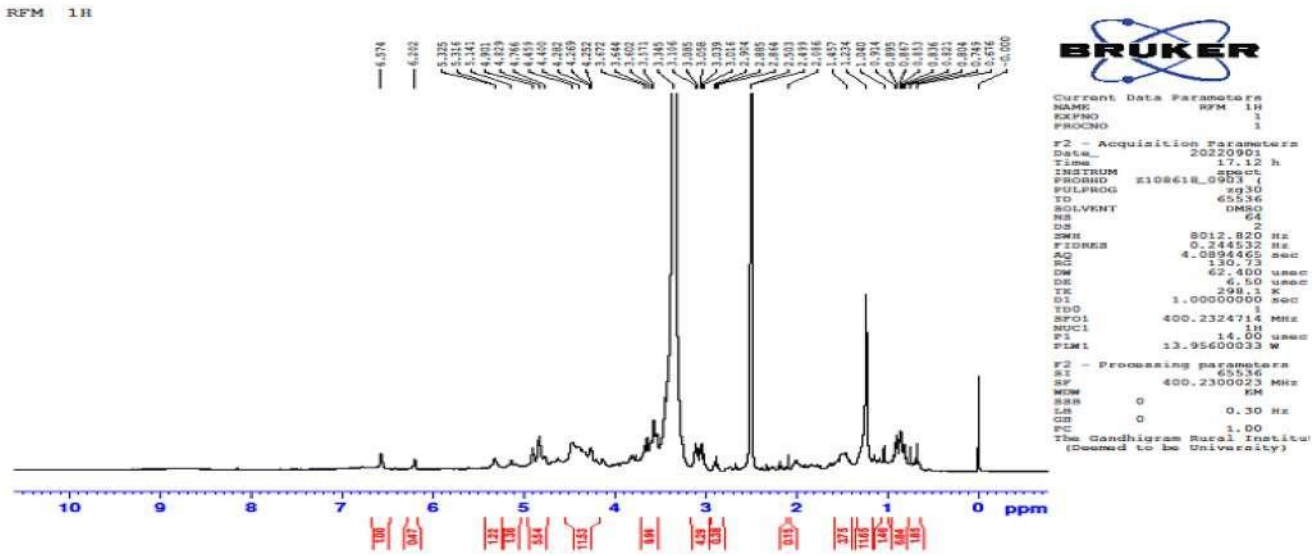


**Figure 8.** NMR analysis of *R. arboreum* Sm methanol flower extract.

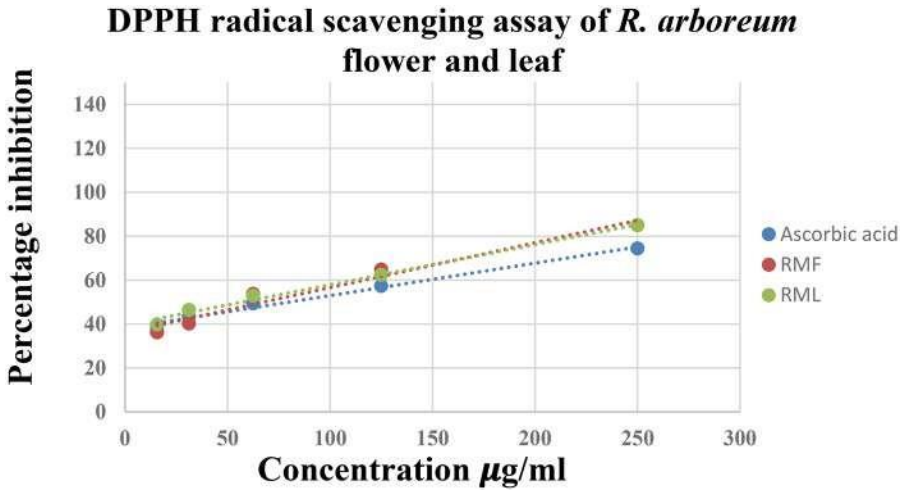


**Figure 9.** DPPH radical scavenging activity of *R. arboreum* leaf and flower.

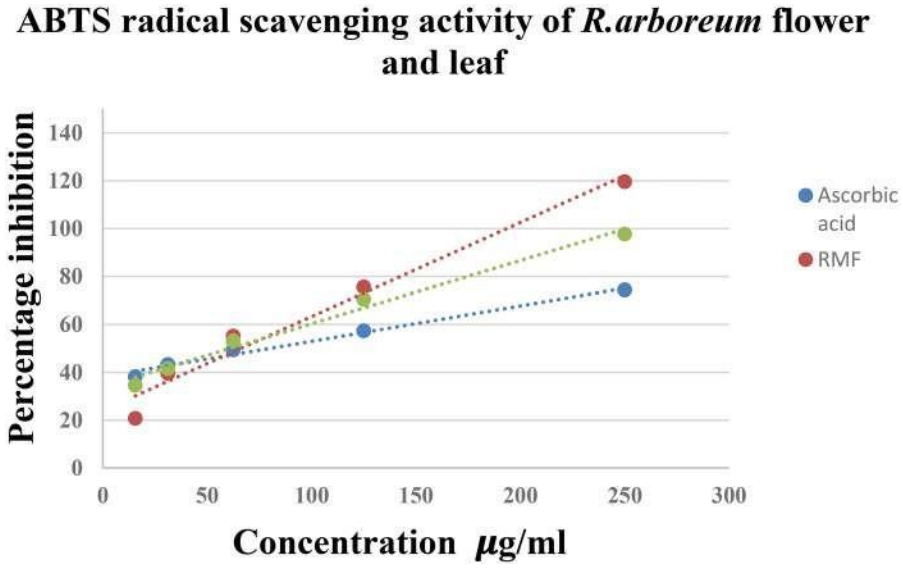


**Figure 10.** ABTS radical scavenging activity of *R. arboreum* leaf and flower.

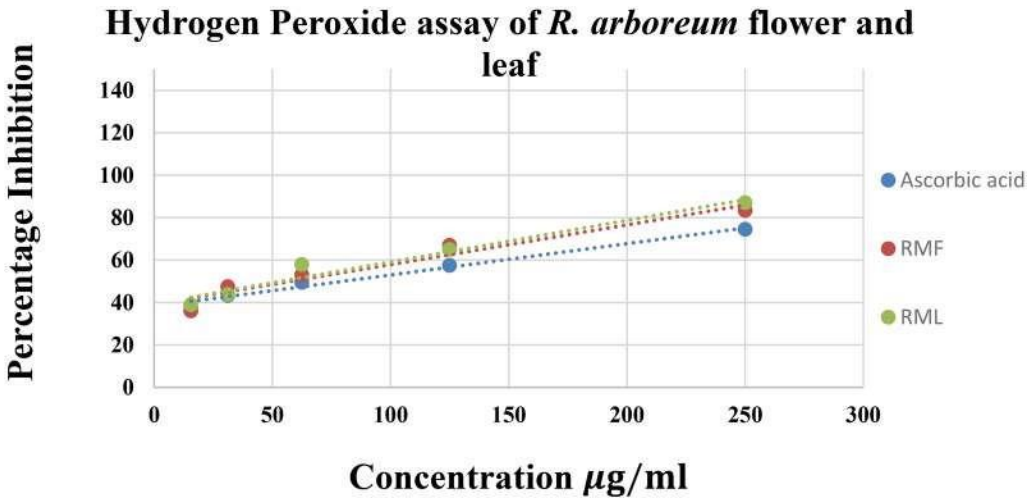


**Figure 11.** Hydrogen peroxide radical scavenging activity of *R. arboreum* leaf.

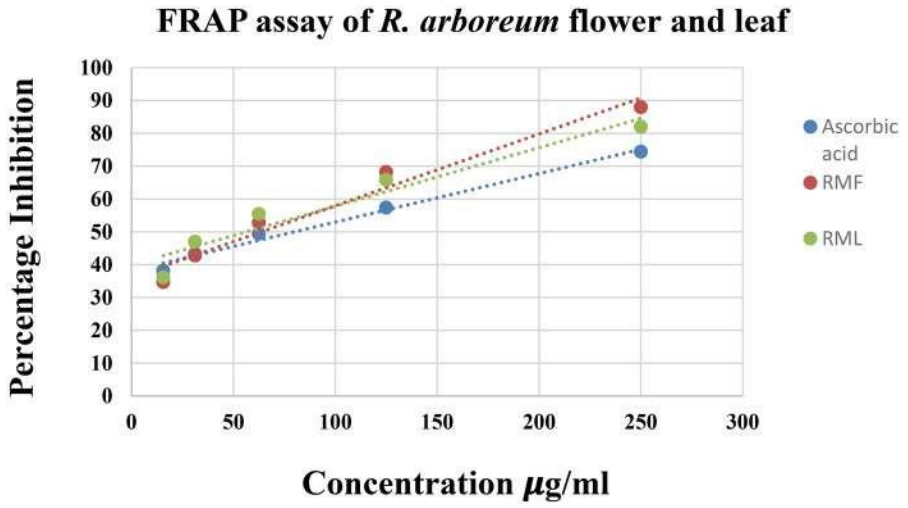


**Figure 12.** Lipid peroxidation assay *R. arboreum* leaf and flower.

50–300 µg/ml. The methanol extract of leaves exhibited an $IC_{50}$ value of 60 µg/mL, while the flower extract showed an $IC_{50}$ value of 65 µg/mL. This indicates that both extracts possess significant antioxidant potential, with the leaf extract demonstrating slightly stronger scavenging activity against ABTS radicals compared to the flower extract (Figure 10). The ABTS assay measures the ability of antioxidants to quench the ABTS radical cation, reflected in a decrease in absorbance at 734 nm.

### *$H_2O_2$ - hydrogen peroxide scavenging activity*

The $IC_{50}$ values for the methanol extracts were found to be 53 µg/mL for the leaves and 57 µg/mL for the flowers. These results indicate that both extracts possess significant antioxidant potential against hydrogen peroxide-induced oxidative stress, with the leaf extract demonstrating slightly higher scavenging activity compared to the flower extract (Figure 11). The hydrogen peroxide scavenging assay measures the ability of antioxidants to neutralize hydrogen peroxide, thereby reducing oxidative damage. The ability of methanol extracts from both the leaves and flowers of *R. arboreum* to scavenge hydrogen peroxide, as indicated by their respective $IC_{50}$ values, was significantly different ($p < 0.05$) compared to the $IC_{50}$ values obtained for the standard compound ascorbic acid.

### *Lipid peroxidation inhibition*

The $IC_{50}$ values for lipid peroxidation inhibition (LPO) by methanol extracts from the leaves and flowers of *R. arboreum* were determined to be 56.6 µg/mL and 60.4 µg/mL, respectively. Both leaf and flower extracts significantly demonstrated higher lipid peroxidation inhibition compared to ascorbic acid alone ($p < 0.01$) tested in egg yolk (Figure 12). These results highlight the superior antioxidant efficacy of *R. arboreum* extracts, suggesting their potential therapeutic utility in preventing oxidative damage associated with lipid peroxidation.

### *FRAP - ferric reducing antioxidant power*

The $IC_{50}$ values for the Ferric Reducing Antioxidant Power (FRAP) assay were determined to be 56 µg/mL for the methanol extract of *R. arboreum* leaves and 63.4 µg/mL for the flower extract. These

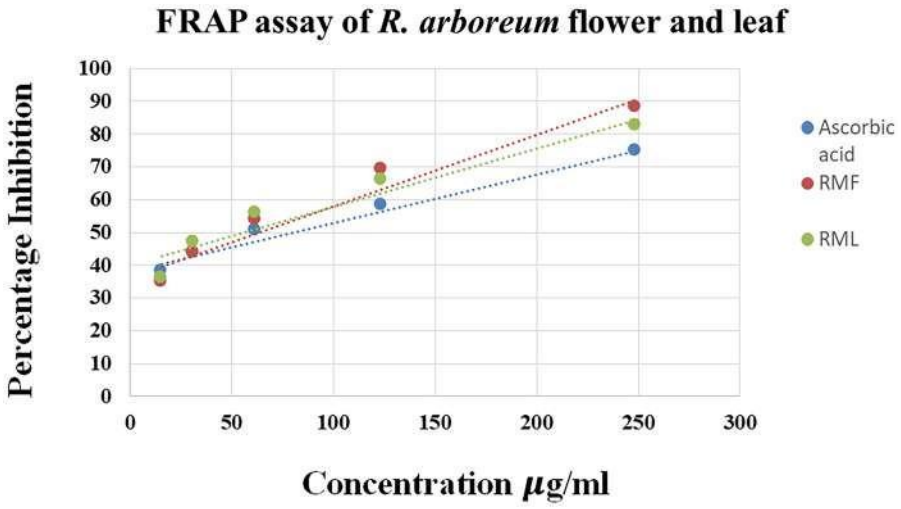


**Figure 13.** FRAP radical scavenging activity of *R. arboreum* leaf and flower.

results indicate that both leaf and flower extracts possess significant ferric reducing antioxidant power, with the leaf extract demonstrating slightly stronger antioxidant activity compared to the flower extract in this assay (Figure 13). The FRAP assay measures the ability of antioxidants to reduce ferric ions ($Fe^{3+}$) to ferrous ions ($Fe^{2+}$), thereby indicating their capacity to donate electrons and neutralize free radicals.

## Discussion

Phytochemicals are chemical constituents found in plants that exert specific physiological effects on the human body [28]. These include alkaloids, carbohydrates, saponins, phytosterols, flavonoids, proteins, amino acids, diterpenes, phenols, tannins, quinones, coumarins, acidic compounds, catechins, volatile compounds, resins, starch, carotenoids, oxalates, and vitamin C. The geographic location of the plant and the choice of solvent used in the extraction process can significantly influence the distribution of these phytochemicals [29].

Rawat et al. [30] conducted a study where qualitative phytochemical analysis of *R. arboreum* leaf extract identified phytosterols, flavonoids, phenols, alkaloids, carbohydrates, glycosides, sterols, steroids, terpenoids, and tannins. However, saponins were absent in all extracts. Similarly, the present study also found saponins absent in all extracts except for the chloroform extract of leaves and distilled water extract of flowers.

Ahmad et al. [31] explored that the methanolic extract of leaves contains flavonoids, steroids, terpenoids, anthraquinones, and tannins. Kiruthiga et al. [32] also documented the presence of phenolic compounds, saponins, proteins, steroids, tannins, xanthoproteins, coumarins, and carbohydrates.

The biochemical composition of *R. arboreum*, focusing on alkaloids, proteins, and carbohydrates. While qualitative analyses have reported the presence of these compounds, quantitative data specific to *R. arboreum* are lacking in the literature. Therefore, our study seeks to fill this gap by employing established methodologies adapted for this plant species. By quantifying alkaloid levels, protein content, and carbohydrate concentrations, we aim to provide comprehensive insights into the biochemical profile of *R. arboreum*. Where our study revealed methanol extracts of flowers exhibited the highest content (32.31 ± 0.03% RMF). Carbohydrate content demonstrated significant differences between leaves and flowers, (73.62 ± 0.13% RAL in leaves and 112.33 ± 0.07% RMF in flowers). Similarly, protein content varied significantly between leaves and flowers, with (79.14 ± 0.01% REF), while leaves generally exhibited lower protein content across all solvents.

In a study by Barola et al. [33], the highest total tannin content (2.90 mg TAE/g) was reported in 30 g of fresh flowers was initially stored at −20 °C for 1 day in a deep freeze and subsequently dried at 110 °C for 25 min, followed by drying at room temperature. In contrast, our study found significant variability in tannin content between leaves and flowers of *R. arboreum*. Leaves exhibited tannin contents of 40.62 ± 0.01% (RML), 28.75 ± 0.02% (REL), and 50.43 ± 0.02% (RAL), while flowers showed 51.57 ± 0.03% (RMF), 80.01 ± 0.16% (REF), and 52.21 ± 0.08% (RAF). These results highlight the distinct tannin accumulation patterns in different plant parts and underscore the influence of extraction methods on tannin quantification.

Rawat et al. [30] conducted a study investigating the total phenolic content (TPC) and total flavonoid content (TFC) of *R. arboreum* using various solvent extracts. They reported TPC values of 1327.5 ± 0.009 mg gallic acid equivalents (GAE)/g, 1468.5 ± 0.006 mg GAE/g, and 1309.5 ± 0.011 mg GAE/g for 100% methanolic, 50% methanolic, and 50% aqueous extracts, respectively. In contrast, our study focused on direct measurements of phenolic and flavonoid content in *R. arboreum's* leaf and flower tissues. We found the leaf phenolic content to be 40.55 mg GAE/g and the flower phenolic content to be 41.92 GAE/g and flavonoid contents of 397.1 mg RE/g in leaves and 398.0 mg RE/g in flowers, with the highest flavonoid concentration observed in the flowers. These findings highlight differences in phenolic and flavonoid concentrations influenced by extraction methods and emphasize the potential medicinal significance of *R. arboreum's* distinct plant parts. This targeted analysis offers clear insights into phenolic distribution within the plant, complementing the broader perspective provided by Rawat et al.'s study [30].

To our knowledge, no prior studies have employed column chromatography to investigate the phytochemical composition of *R. arboreum*. While high-performance liquid chromatography (HPLC) methodologies have been documented in the literature, our study represents the first utilization of column chromatography with silica gel (60–120 mesh) for this plant species. This technique allows for the separation of compounds based on their polarity differences, yielding 17 distinct fractions from *R. arboreum*

leaves and 24 from flowers. Each fraction is characterized by specific $R_f$ values, indicating varying degrees of compound mobility within the chromatographic system.

In our study, *R. arboreum* leaves yielded fractions with $R_f$ values ranging from 0.06 to 0.93, reflecting a diverse array of compounds. The presence of multiple $R_f$ peaks in certain fractions suggests the presence of complex mixtures. Conversely, *R. arboreum* flowers exhibited distinct $R_f$ values clustered around 0.1, 0.76, 0.85, and 0.9, highlighting unique chemical profiles within different fractions. Specially, fractions 12 and 13 from flower extracts did not exhibit detectable spots under the conditions tested, indicating the potential absence of UV-absorbing compounds in these fractions.

By initiating the use of column chromatography in *R. arboreum* phytochemical analysis, our study opens new avenues for exploring the chemical diversity and bioactive potential of this plant species. The identified fractions can serve as starting points for further isolation and characterization of specific compounds, offering insights into their pharmacological, nutritional, and ecological roles.

In a previous study, Thin Layer Chromatography (TLC) analysis of *Rhododendron* flower extract revealed the separation of six major fractions, identifiable as distinct bands under UV transillumination at 254 and 366 nm [34]. In contrast, our study expanded upon this approach by analyzing *R. arboreum* flower and leaf samples using TLC, yielding a broader range of $R_f$ values indicative of diverse chemical components.

To explore additional phytoconstituents in *R. arboreum* leaves and flowers, we utilized GC-MS analysis. Our findings revealed common presence of linoleic acid, flavone 4′-OH, Benzenepropanoic acid, hexadecanoic acid (CAS), palmitic acid, and 9,12-Octadecadienoic acid in both leaves and flowers. These compounds span various categories; for instance, palmitic acid and linoleic acid are classified as fatty acids. Palmitic acid, known for its antioxidant properties, exhibits potential as a free radical scavenger, highlighting its significance in biological systems [35].

In our study, we employed FTIR spectroscopy to analyze leaf and flower extracts of *R. arboreum,* revealing significant absorption peaks indicative of diverse chemical constituents. The leaf extract exhibited prominent peaks at wavelengths such as 3880.78 $cm^{-1}$ (O-H stretching, phenols), 3387.00 $cm^{-1}$ (N-H stretching), 2924.09 $cm^{-1}$ (C-H stretching, aliphatic hydrocarbons), 2306.86 $cm^{-1}$ (C≡N stretching), and 1689.64 $cm^{-1}$ (C = O stretching, ketones/aldehydes), consistent with findings in previous studies (Phuyal et al. 2020). Similarly, the flower extract showed distinct peaks at 3718.76 $cm^{-1}$ (O-H stretching, phenols), 3286.70 $cm^{-1}$ (N-H stretching), 2924.09 $cm^{-1}$ (C-H stretching, aliphatic hydrocarbons), 2314.58 $cm^{-1}$ (C≡N stretching), and 1689.64 $cm^{-1}$ (C = O stretching, ketones/aldehydes), aligning with observations related to the involvement of phenolic acids in nanoparticle formation [36]. Our findings corroborate those reported by Ahir et al. [37] prominent peaks at 3430 $cm^{-1}$ (OH stretching vibrations) in the petal extract were identified. These results underscore the consistent presence and potential role of these functional groups from *R. arboreum*.

The NMR findings from Shagun et al. [38], which identified quinic acid and chlorogenic acid in hot aqueous extracts of *R. arboreum*, align with our study's 1H-NMR spectroscopy results, which characterized the broader chemical composition of these extracts in detail. Shagun et al. [38] demonstrated the presence of specific acids, providing a foundational understanding of key constituents in *R. arboreum*. In our study, we extended this analysis by identifying distinct proton signals across various spectral regions: deshielded protons in aromatic or vinylic systems (6.674–6.171 ppm), protons adjacent to electronegative groups or within conjugated systems (5.436–4.172 ppm), contributions from methylene groups or complex functionalized groups (3.714–2.951 ppm), and signals from protons near electron-withdrawing groups or specific functional groups (2.940–2.075 ppm). Moreover, the presence of significant integrals in the aliphatic region (1.576–0.631 ppm) indicated the abundance of methyl and methylene groups, with distinct signals from aliphatic chains (1.337–1.174 and 0.961–0.631 ppm). Together, these findings deepen our understanding of the chemical diversity within *R. arboreum* extracts.

We evaluated the antioxidant activity of dry leaf and dry flower extracts of *R. arboreum* using the DPPH assay. Our findings showed that the $IC_{50}$ values for *R. arboreum* dry leaf and dry flower extracts were 56 µg/mL and 67 µg/mL, respectively. These results indicate potent antioxidant capabilities for both extracts. This contrasts with a previous study reporting higher antioxidant activity in *R. arboreum* dry leaf ($IC_{50}$ = 24.65 ± 1.47 µg/mL) compared to dry flower (42.34 ± 1.13 µg/mL) and fresh flower (48.15 ± 1.65 µg/mL) extracts [39]. Our focused evaluation underscores the strong antioxidant potential of *R. arboreum* dry leaf and dry flower extracts.

The study reported by sharma et al. [4] on the methanolic extract of *R. arboreum* flowers had the highest ABTS radical activity with an $IC_{50}$ of 11.49 ± 1.25 µg/mL, indicating potent antioxidant capability. In contrast, our study found that the methanol extract of *R. arboreum* leaves exhibited an $IC_{50}$ value of 60 µg/mL, while the flower extract showed an $IC_{50}$ value of 65 µg/mL. These suggest variability in antioxidant activity depending on the plant part (leaves vs. flowers) and possibly extraction methods used for the study.

No previous studies have reported on the hydrogen peroxide ($H_2O_2$) scavenging assay for *R. arboreum*, prompting our investigation into this aspect. Our study revealed $IC_{50}$ values of 53 µg/mL for the methanol extract of leaves and 57 µg/mL for the methanol extract of flowers. The $H_2O_2$ scavenging assay assesses the ability of antioxidants to neutralize hydrogen peroxide, thereby protecting cells from oxidative damage [40].

The study by Acharya et al. [41] reported potent inhibition of lipid peroxidation by the ethanolic extract of *R. arboreum*, with an $IC_{50}$ value of 45.6 µg/mL. In alignment with their findings, our study investigated methanol extracts from *R. arboreum* leaves and flowers for lipid peroxidation inhibition (LPO). We found $IC_{50}$ values of 56.6 µg/mL for the leaf extract and 60.4 µg/mL for the flower extract. This aligns with the findings of Acharya et al. [41], demonstrating that *R. arboreum* extracts, regardless of the extraction solvent, possess valuable antioxidative properties against lipid peroxidation.

The study by Kashyap et al. [42] reported a high FRAP assay value of 140.6 ± 2.76 mM TE/g for *R. flower* extract, attributing this antioxidant activity to polyphenols present in *R. arboreum* flowers and their effective free radical scavenging capacity. In alignment with this, our study determined $IC_{50}$ values for the FRAP assay as 56 µg/mL for the methanol extract of *R. arboreum* leaves and 63.4 µg/mL for the flower extract. These results collectively underscore the potent antioxidant potential of *R. arboreum* extracts, corroborating their ability to effectively reduce ferric ions and scavenge free radicals, thereby supporting their therapeutic potential in combating oxidative stress-related disorders.

## Conclusion

The qualitative phytochemical analysis of *R. arboreum* extracts revealed a diverse array of bioactive compounds including alkaloids, carbohydrates, saponins, phytosterols, flavonoids, proteins,

amino acids, diterpenes, phenols, tannins, quinones, coumarins, acidic compounds, catechins, volatile compounds, resins, starch, carotenoids, oxalates, and vitamin C. Further analytical methods such as column chromatography, TLC, GC-MS, FTIR, and NMR provided detailed insights into the chemical composition and structure of these compounds. Bioactivity assays demonstrated significant antioxidant potential in both leaf and flower extracts, as evidenced by their ability to scavenge DPPH, ABTS, and hydrogen peroxide radicals, inhibit lipid peroxidation, and exhibit ferric reducing antioxidant power. These findings underscore the therapeutic potential of *R. arboreum* as a source of natural antioxidants with possible applications in health and medicine.

## Authors' contributions

**Yangchen Dolma Kom:** Planned the work and carried it out, wrote the manuscript.

**R. Karthiyayini**: Ph. D supervisor, guidance throughout the completion of work and manuscript.

**Surya Suresh:** Minor correction in manuscript.

## Ethics approval and consent to participate

Not Applicable.

## Consent for publication

All authors have read and approved the final manuscript.

## Disclosure statement

No potential conflict of interest was reported by the authors.

## Availability of data and materials

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

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## ORCID

Yangchen Dolma Kom http://orcid.org/0000-0003-2751-336X
Karthiyayini Ramaswamy http://orcid.org/0000-0002-2305-3581
Surya Suresh http://orcid.org/0000-0002-3129-9151

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REVIEW ARTICLE

# A Review: *Ophiocordycep Sinensis* (Berk.) as a Traditional Tibetan Medicine and its Potential in the Treatment of Various Human Ailments

Yangchen Dolma Kom[1] and R. Karthiyayini[1,*]

[1]*Department of Botany, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore -43, Tamil Nadu, India*

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**Abstract:** Nature's never-ending gift, which counts as one of the most valuable medicines in the entire world, the *Ophiocordyceps sinensis*, also known as caterpillar fungus, is a traditional Chinese medicine found in Tibetan plateaus. During the season of spring, many shepherd search for this cat- erpillar fungus as its value in the market is very high. Moreover, due to the effects of climatic change on its population and increasing demand, *O. sinensis* is now an endangered species. It can treat almost many diseases, such as cardiovascular disease, respiratory diseases, immune dysfunc- tion etc. The chemical composition of this fungus includes amino acids, glutamic acid, polyamines, cordycepic acid, saccharides, sterols, nucleotides, sugar derivatives, fatty acids, vitamins, and oleic acid.

**Keywords:** Antioxidant, anti-tumor, immune dysfunction, amino acid, *Ophiocordycep sinensis*, vitamin.

## 1. INTRODUCTION

### 1.1. *Ophiocordyceps sinensis* (YARTSA GUNBU)

Nature has provided us with abundant resources that are essential for mankind, and most importantly, medicines are the only hope of living beings to rely on. *Ophiocordycep sinensis* (Yartsa gunbu) in the Tibetan language is a winter worm/summer grass also known as caterpillar fungus be- longing to the family of Hepialidas. It lives under the soil burrows of ground in the Tibetan Plateau, where it feeds on other plant roots as a host insect to survive. Eventually, the worms get infected by a fungus during summer when they are buried under the soil, and the fungus starts invading the host body of the caterpillar while consuming it slowly dur- ing the winter. As the fungus grows vegetatively in the cat- erpillar's body, it fills it with threadlike hyphae, infecting ghost moth larvae within the family Hepialidae during the autumn (Fig. **1**). The larvae, even when infected by the fun- gus, can still migrate from 5-25 cm below the soil surface to 2-5 cm beneath before dying [1]. *Ophiocordycep sinensis* has been used for traditional Chinese remedies in the treatment of diseases like respiratory inflammation, asthma and many more [2]. Also, in 2003, it was reported that SARS could be cured with caterpillar fungus. Native people of Tibet region consume it as an immune booster. This medicinal activity is due to the parasitized fungus, and both the fungus and cater- pillar, which remain connected, should not be separated (Fig. **2**). The areas where these caterpillar fungi are present have thick soil. Hence, harvesting and digging them out of soil is very difficult as the value of this caterpillar fungus goes down if the fungus and caterpillar are not attached. However, their distribution is very limited to the Tibetan plateau geographic area of Chamdo and Yushu [3]. Due to the high medicinal potential of Yartsa gunbu, the price in- creases rapidly by about $140,000 per kilogram, which also results in the fluctuation of their population. The reason could be high commercial demand and the climatic change where extreme cold conditions (-5°C) are required to parasi- tize the fungus and the caterpillar. However, it is believed that climatic change has been a major disturbance in nature for the winter worm [4].

## 2. SYSTEMATIC CLASSIFICATION

| | |
|---|---|
| Kingdom | Fungi |
| Division | Ascomycota |
| Class | Sordariomycetes |
| Order | Hypocreales |
| Family | Ophiocordycipitaceae |
| Genus | *Ophiocordyceps* |
| Species | *Sinensis* |

*Address correspondence to this author at the Department of Botany, Avi- nashilingam Institute of Home Science and Higher Education for Women, Coimbatore -43, Tamil Nadu, India; Tel: 9944022592; E-mail: karthi- sai13@gmail.com

 

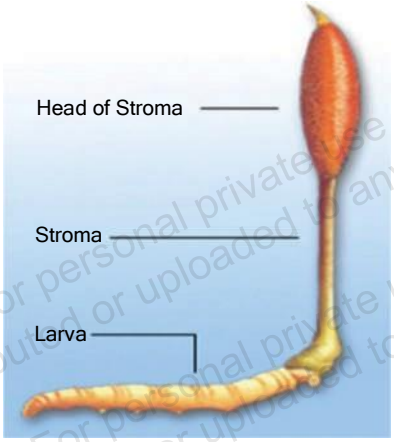


**Fig. (1).** *O. sinensis* body parts. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

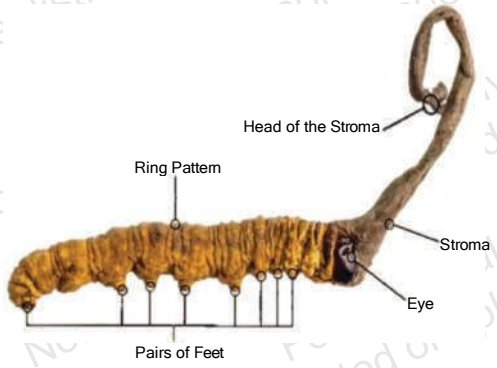


**Fig. (2).** Stroma emerging from the head. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

## 3. REPRODUCTION

*Ophiocordycep sinensis* is a fungus that grows and reproduces naturally on host insects, but there are over fifty species of insects in the family Hepialidae that host it. The fungus also takes roughly 2 to 3 years to produce a fruit, which is why growing and reproducing the fungus requires hosts [1]. Spring is the time when the stroma bud grows upwards and forms a stalked fruiting body containing (asexual) mating-type genes [5]. Ascomycota fungi have a single mating type locus (MAT) that controls sexual development [6]. A mating-type locus is characterized by two alternate forms (MAT1-1 and MAT1-2), which are called idiomorphs instead of alleles [7]. Genes that are of the mating type evolve at a faster rate than ITS (Internal transcribed spacer) and glyceraldehyde 3-phosphate dehydrogenase sequences [8, 9].

### 3.1. Species and Distribution

Almost 50 species from the family of Hepialidae have been discovered and identified throughout the main producing regions of *Ophiocordyceps sinensis*, of which twelve are reported from Sichuan, twenty from Yunnan, nine from Qinghai, fourteen from Tibet, and three from Gansu [10]. The biological characteristics of almost more than ten spe- cies of genus insects have been well considered for the study in China, which include H. armodcamu Oberthar, H. minyuancus, H. yushusis Chuet Wang, H. lagii Yan, H. gonggaensis, H. guidera Yan, H. oblifurcus Chuet Wang, H. renzhiensis, H. deginensis, H. alttdcoh W, H. biru , H. pui sp. nov., and Thitarodes pui [11]. As the host insects of *Ophiocordyceps sinensis* tend to be regionally and vertically distributed, they can be found mainly in the Qinghai-Tibetan Plateau zones. There are specific geographical locations and patterns of distribution for each insect population [12]. It is observed that most are located above 4000 meters above sea level. Among the different zones, the most wide-ranging vertical distributions are found in Tibet, followed by Si-chuan and Yunnan, and the smallest in Qinghai and Gansu. A host insect lives in soil except during its adult period, and its eco-distribution is influenced by many factors, such as climate, terrain, landform, altitude, vegetation, and agro-type [13]. Food-stuffs and soil microclimate play a vital role in determining host insect distribution in the region. Insects of the Hepialus species can be found everywhere in alpine, sub-alpine, and alpine shrubs, whose soil conditions are either alpine meadow soil or sub-alpine meadow soil [14].

### 3.2. Artificial Rearing of Host Larvae- Hepialus

The Hepialus of *Ophiocordyceps* needs three to four years to complete their work. The larval stage takes about two years to complete under the circumstances of artificial rearing [15]. The artificial culture of Hepialus host larvae in a soilless environment requires growth nutrition, and disease prevention would be the fundamental strategy for implementing the artificial cultivation of *Ophiocordyceps sinensis*. The artificial formula for feeding, which includes Rheum pumilum 5.0, sorbic acid 0.5, soybean 15, maize flour 10, wheat bran 8, yeast powder 4.5, agar 10, and water 100g/L, can provide the nutritional components for the larvae. The natural Rheum pumilum plants can be used to increase the weight of the larvae, while the manufactured feeds can contain antiseptics, such as sorbic acid and nipagin esters, that would not stunt the larvae's growth [12].

### 3.3. Segregation of Fungal Strains

A reasonably straightforward operational procedure is used to first separate the fungus from the *Ophiocordyceps sinensis* components. This fungus may be isolated from a variety of *Ophiocordyceps sinensis* tissues and organs at various stages of development, which include fruiting body, mummified *O. sinensis*, live larvae, and ascospore [16].

### 3.4. Germination of Natural Fruiting Body in Correspondence to Ascospore

The mummified caterpillar is buried in the ground and is typically used as a raw material. First, alcohol is typically used to sanitize the materials' surfaces. The materials are then meticulously inserted into water agar media and grown at 20°C. The material for the experiment is then chosen to be a natural fruiting body in correspondence to ascospore; the typical isolation approach involves sterilizing the fruit- ing body along ascospore, hanging two extremes stroma over medium, and inclining the plane of potato dextrose agar (PDA) in cuvette. They are grown at 20°C once the spores land on the medium and start to germinate [12].

### 3.5. Strains Derived from Ascospore

The larva's body surface is then severed after sterilizing. After that, the bodily fluids are grown at 20°C. Individual hyphal ends are cut and placed on PDA media once the major clone emerges. The mycelium's shape is then studied and recognized. Due to the separation of the two strains, the isolation process is crucial for the development of the fruiting body [17]. The strains isolated from the fruiting body are significantly easier to generate the fruiting bodies in comparison to other places, such as the stroma petiole and sclerotium. Additionally, strains produced through ascospores have an advantage over those obtained from other tissues in terms of producing fruiting bodies [12].

### 3.6. Medicinal Importance

This valuable herb is likely found in the Tibetan plateau, India, Bhutan, and Nepal as the most prized commercial medicine [18]. However, due to climatic change and increased demand, its production has decreased. As a result, its price has also rapidly increased. Moreover, the medicinal fungus was also mentioned in Ayurvedic Literature which is known to be one of the ancient works of literature on herbal wild medicines. It tells us about the knowledge of life, in which this fungal medicine was mentioned as Bhu-Sanjivani [19]. It has a cure for several incurable diseases, such as cancer, asthma, and sexual dysfunction. This medicinal fungus is consumed as a tonic in alcohol, water, and milk by the native people of Tibet to boost anti-tumor activity and immunity. Moreover, due to the presence of chemical constituents, including amino acid, glutamic acid, polyamines, cordycepic acid, saccharides, sterols, nucleotides, sugar derivatives, fatty acid, vitamins, and oleic acid, it has various pharmacological activities, such as improving the respiratory system and physical performance [20]. Reports and evidence have also proved that the cordyceps polysaccharides are known for bringing effective improvement in our immune system, which also protects our liver from hyperlipidemia.

#### *3.6.1. Stamina*

It is believed that an athlete named Wang Jungxia, who made the 10,000m world record in just forty-two seconds in 1993 and enjoyed 23 years of fame, was the next Junren Ma. When this story trolled out among people, Ma was dropped from the Olympic game because he failed the drug test due to consuming Cordyceps product, which helped maintain blood glucose during prolonged workouts[1]. Research has concluded that consumption of these *cordyceps senensis* increases cellular bio-energy-ATP (adenosine triphosphate); in this, the synthesis of ATP is faster than usual, and the energy spent gets reloaded very rapidly with an improvement of oxygen utilization and anti-fatigue effects.

#### *3.6.2. Tonic for Liver*

By consuming Yartsa gunbu, one can improve liver function and reduce the inflammation in the liver. It actually increases serum component levels in posthepatic cirrhosis patients and regulates cellular immune functions. Cordyceps polysaccharides (CPs) are the bioactive compounds that protect the liver. CPs help in improving the immunological functions of the organic cells with the help of the removal of harmful components and hence resulting in the reduction of injury to the liver cells. The most famous and sacred recipe of Fuzheng Huayu helps in the prevention or suppression of the development of post-hepatic cirrhosis for further complications [21].

#### *3.6.3. Anti-tumor Activity*

Yartsa gunbu has a very special constituent of cordyglucan present on the polysaccharide cell wall of the species, which is believed to have the potential to exhibit antitumor activities [21]. Its polysaccharide exerts an anti-tumor effect by developing an immune response against the tumor-bearing cells, apoptosis of tumor cells by direct anti-tumor activity, and oral administration of polysaccharide which is isolated from the *Ophiocordycep senensis* helps in prevention of oncogenesis and stops the spread of tumor cells in other parts of the body [22]. Enhancement of immunological function and inhibition of RNA synthesis affects the synthesis of proteins when apoptosis of tumor cells continues by regulating the signal pathways [23], antioxidant free radical scavenging activity, and anti-mutant activity by preventing the replication of tumor cells and nuclei acid methylation.

#### *3.6.4. Immune System*

The tonic is widely used to nourish the body and brain, increase appetite, as well as memory and endurance and address the issue of insomniacs. The immune-stimulating and immune-suppressive activities of IPSs and EPSs are evaluated with the aid of natural killer cells, including T cells, B cells, and macrophages [24]. Proinflammatory cyto- kine production, which is controlled by macrophages, inhib- its the invasion of pathogens [25]. Based on evidence that the heteropolysaccharide limits oxidative damage and con- trols IL-4, IL-5, and IL-17, the heteropolysaccharide has a positive impact on immune activity in mice exposed to ion- izing radiation [26]. (24R)-ergosta-7,22-dien-3â,5á,6â-triol inhibits the production of IL-2 by monocytes and lymph nodes in renal failure, including IgA nephritis, autoimmune nephritis, and lupus nephritis [27].

#### *3.6.5. Respiratory Function*

Yartsa gunbu has a stimulatory effect on ion transport in the epithelial cell due to the presence of cordycepin and adenosine. It enhances the capacity of oxygen utilization. This is how people residing at high altitudes fight against the illness. *C. sinensis* plays an important role in the secre- tion of adrenaline from adrenal glands and inhibits the contraction of the tracheal through histamine, which is very important for airflow in the lungs for asthma patients. It also prevents pulmonary emphysema and has anti-asthmatic action. Moreover, tt is used in healing many respiratory diseases [28].

### *3.6.6. Cardiovascular Functions*

The use of cordyceps in combination with *O. sinensis* and cyclosporine therapeutics has been proven to reduce acute, chronic rejection in cardiac transplant patients, which assists in suppressing acute rejection [29].

### *3.6.7. Anti-hyperglycemia*

A common noncommunicable disease among poor countries is diabetes mellitus. One study based on *O. sinensis* [30] used the crude polysaccharide of the *O. sinensis* to inject into normal mice and streptozotocin to inject into induced diabetic mice [31]. After prolonged administration, both normal and streptozotocin diabetic mice showed immediate low plasma glucose levels resulting in lowered glucose levels with oral feeding. Using animal models caused by alloxan and streptozotocin (STZ), numerous researchers have already assessed the impact of natural products, including fungal polysaccharides [32], on hyperglycemia.

### *3.6.8. Antioxidant Activity*

By identifying and isolating natural antioxidants from plants, marine algae, and fungi, we can manufacture the best nutraceuticals for use in functional food to improve health and prevent disease [33]. In a study, this was done by using the column chromatography purified polysaccharide en- riched fraction, which was isolated from these *Ophio- cordyceps senensis*, which resulted in the strong presence of oxidative activities. Then, CPS-1polysaccharide was a com- pound with MW 210 kDa, and derived through activity- guided fractionation, was found to be the strongest antioxi- dant against rat PC12 pheochromocytoma cell damage caused by $H_2O_2$, lipid peroxidation, and oxidative stress. Also, the cell activates various major antioxidant enzymes, including superoxide dismutase [34]. In the isolation of the APS from *Tolypocladium senensis*, another anamorph of *Ophiocordyceps senensis*, the APS was found to be crucial in protecting PC12 cells from $H_2O_2$-induced cells that damage the cells by increasing glutathione peroxidase and superoxide dismutase [35]. A black pigment derived from melanin was isolated from the fermented broth of *Ophiocordyceps senensis*, which was highly scavenging, thanks to its antioxidant activity and reduced malondialdehyde levels (MDA). 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Tables **1** and **2**).

**Table 1. Some major Pharmacological activities of compounds from *Ophiocordycep sinensis.***

| Compounds | Pharmacological Activities | | | | | | | | | References |
|---|---|---|---|---|---|---|---|---|---|---|
| | Anti-cancer | Aphrodisiac | Anti-inflamatory | Anti-oxidant | Anti-asthemia | Hypolipidemic | Immunomodulatory | Hyocholesterolem | Anti-hypertension | |
| Cordycepin | + | + | + | + | + | - | ↓ | - | + | [2] |
| Ergosterol | - | - | - | - | - | - | - | - | - | [14] |
| Ergosterolperoxide | + | - | + | - | - | - | - | - | - | [30] |
| β-sitosterol | + | + | - | - | - | - | ++ | - | - | [31] |
| Stigmasterol | - | - | + | - | - | - | - | + | - | [32] |
| Campesterol | - | - | - | - | - | - | - | + | - | [33] |
| Adenosine | - | - | - | + | + | - | - | - | - | [34] |
| Cordyceamides | + | - | - | - | - | - | - | - | - | [35] |
| Cordymin | - | - | + | + | - | - | - | - | - | [36] |
| Cordycedipeptide | + | - | - | - | - | - | - | - | - | [37] |
| D-mannitol | - | + | - | - | + | - | - | - | + | [38] |
| Butylated hydroxytoaline | - | - | - | + | - | + | - | + | - | [39] |

**Note:**
Where,
= Absence of Activities, + = Presence of compound,
++ = Strongly Presence of activities, ↓ = Immunosupression.

**Table 2. Bioactive ingredients and bioactivities, and material source of *O.sinensis.***

| No. | Bioactive Ingredient | Bioactivities | Material Source | References |
|---|---|---|---|---|
| 1 | Ergosterol | Cytotoxic | Mycelium | [11] |
| 2 | Sitosterol | Cytotoxic | Mycelium | [11] |
| 3 | Serine protease | Fibrinolytic | Culture supernatant | [40] |
| 4 | Cordycepin | Steroidogenesis, antimetastatic activity,antitumor, immunomodulatory | Culture supernatant | [41] |
| 5 | Cordymin | Antioxidant, anti-inflammation | - | [42] |
| 6 | Intracellular polysaccharides | Immunostimulatory, antitumor, immunomodulatory, antioxidant, protection of chronic renal failure, cholesterol esterase inhibitory activity | Mycelium and fruiting body | [43-47] |
| 7 | Extracellular polysaccharides | Immunomodulatory and antitumor, antioxidant | Culture supernatant | [48-53] |
| 8 | Adenosine | Immunomodulatory | Mycelium | [54] |
| 9 | Guanosine | Immunomodulatory | Mycelium | [54] |
| 10 | Lovastatin | Hypolipidemic | Mycelium | [55] |
| 11 | γ-aminobutyric acid (GABA) | Neurotransmitter | Mycelium | [56] |
| 12 | Ergosta-4,6,8(14),22-tetraen-3-one(ergone) | Cytotoxic | - | [57] |
| 13 | 5α,8α-epidioxy- 22E-ergosta-6,22-dien-3β-ol | Cytotoxic | Mycelium | [58] |
| 14 | 5α,8α-epidioxy-22E-ergosta-6, 9(11),22-trien-3β-ol | Cytotoxic | Mycelium | [29] |
| 15 | 5α,6α-epoxy-5α-ergosta-7,22- dien-3β-ol | Cytotoxic | Mycelium | [59] |
| 16 | 5α,8α-epidioxy-24(R)-methylcholesta-6,22-dien- 3β-D- glucopyranoside | Antitumor | Mycelium | [60] |
| 17 | 5,6-epoxy-24(R)-methylcholesta-7,22-dien-3β-ol | Antitumor | Mycelium | [60] |
| 18 | Myriocin | Immune inhibitor | - | [61-63] |
| 19 | Melanin | Antioxidant | Mycelium | [64-69] |

## CONCLUSION

The caterpillar fungus was renamed in 2007 from *Cordyceps sinensis* to *Ophiocordycep sinensis*. These fungi are highly valuable in the market for their high importance in healing many human ailments. Moreover, polysaccha- rides present in this mushroom are an active component with almost 20 bioactive compounds, which promote health and can prevent many diseases, such as heart-related diseas- es, respiratory diseases, and many more.

## AUTHORS' CONTRIBUTIONS

Dr. R. Karthiyayini, Ph. D supervisor, provided guidance throughout the completion of the review paper on *O. sinensis*.

## LIST OF ABBREVIATIONS

ATP = Adenosine Triphosphate

CPs = Cordyceps Polysaccharides

ITS = Internal Transcribed Spacer

## CONSENT FOR PUBLICATION

Not applicable.

## FUNDING

None.

## CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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